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# The Journal of Clinical Investigation

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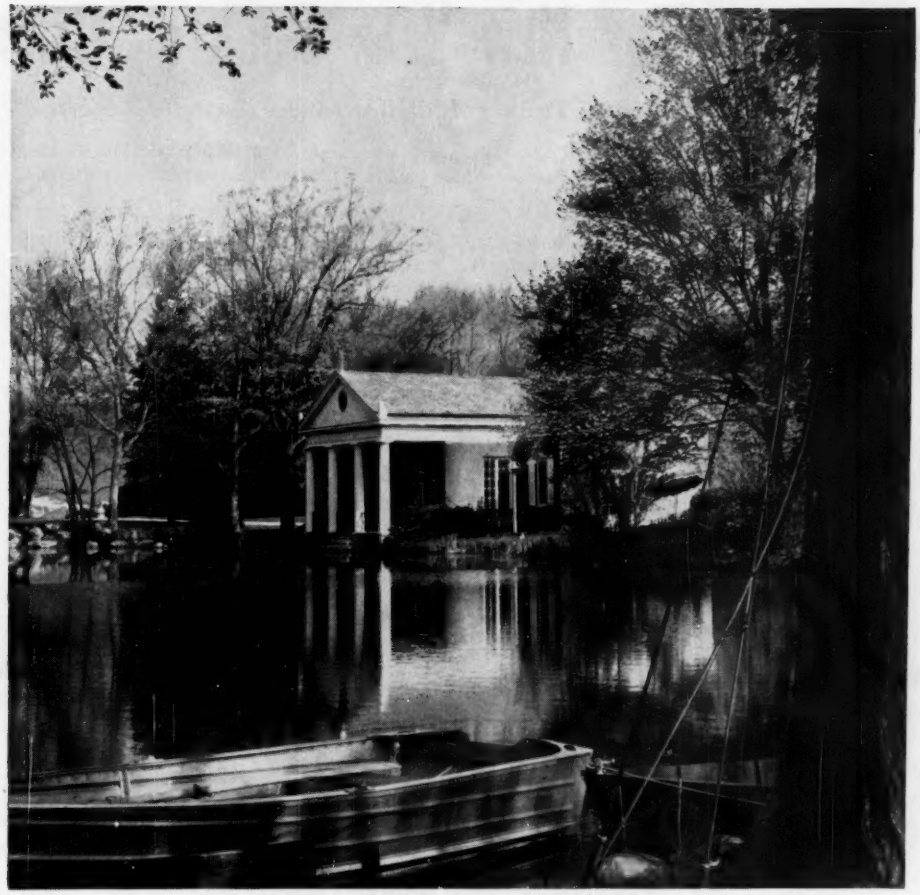

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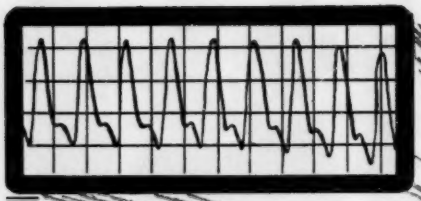

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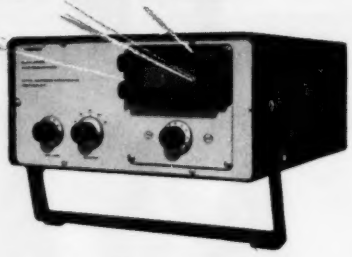

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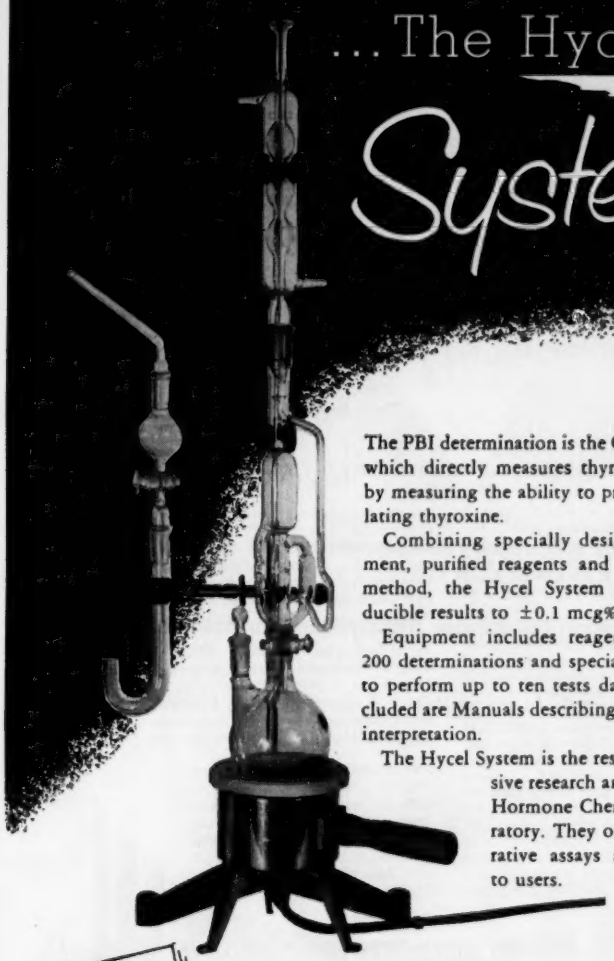

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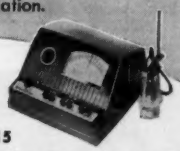

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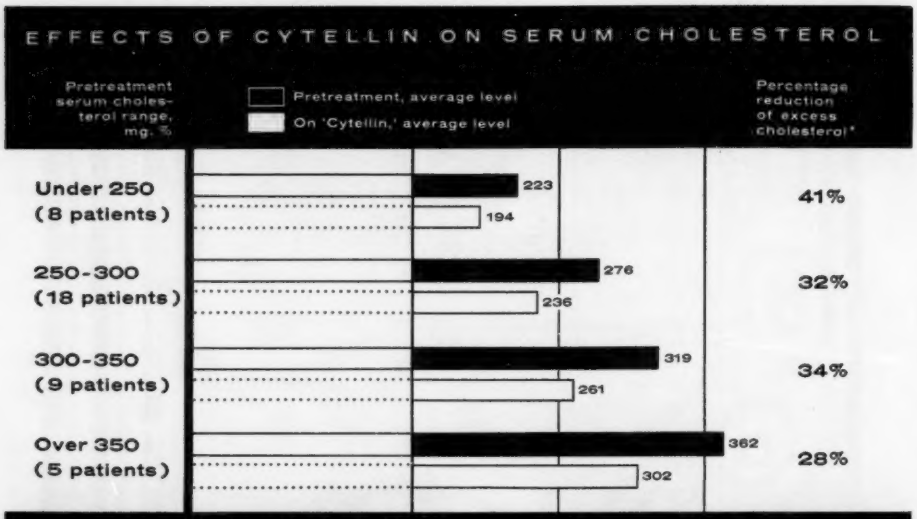


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# THE INFLUENCE OF BODILY HYDRATION ON THE RENAL CONCENTRATING PROCESS[1]

By FRANKLIN H. EPSTEIN,[2] CHARLES R. KLEEMAN, AND ACHIEL HENDRIKX

*(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.)*

(Submitted for publication December 7, 1956; accepted December 28, 1956)

Although the ability of the kidneys to produce a concentrated urine is widely used in the clinic as a measure of renal function, there have been few systematic studies of factors affecting maximum urinary concentration in normal persons. It is clear that a major influence on renal reabsorption of water is exerted by the antidiuretic hormone of the posterior pituitary (ADH); however, Shannon pointed out that, even in the absence of exogenous or endogenous Pitressin®, dogs with diabetes insipidus could elaborate a concentrated urine when they were severely dehydrated (1). It has been observed repeatedly that injecting large amounts of posterior pituitary substance into hydrated normal subjects does not produce as concentrated a urine as does prolonged dehydration (2–6).

The present experiments were designed to explore some of the effects of bodily hydration in altering the response of normal kidneys to maximum doses of ADH. The results indicate that chronic underhydration as well as prolonged forcing of fluids drastically modify the ability of the kidneys to reabsorb water and to concentrate solutes in the urine.

## METHODS

Normal young men subsisted for three days on a) a *Dehydration diet* containing no water or liquid food, b) an *Ad lib* regimen in which the same food was taken, but no restriction was placed on drinking, or c) an *Overhydration* regimen, which differed from the preceding only in that 5 to 6 liters of water were imbibed in divided doses throughout the day. Each individual served as his own control by participating in at least two experiments of different types. In some subjects the sequence was repeated in exactly the same way, but the protein content of the diet was varied. Results of varying the intake of protein will be discussed in detail separately (7). On the morning of the fourth day the subject reported to the laboratory, having abstained from food and fluids for the preceding 12 hours. In order to assure a constant and maximal antidiuretic stimulus, Pitressin®[3] was infused throughout all experiments at the rate of 200 milliunits per hour in 50 cc. of 0.9 per cent saline containing inulin. This dose of Pitressin® is approximately four times the amount needed to produce a maximally concentrated urine in normal human subjects (8). Subjects remained in the supine position, standing only to void every half-hour or hour. After two to three hours, when urinary osmolality had reached a plateau, 10 per cent mannitol was infused intravenously at 20 cc. per min. into some subjects without interrupting the administration of Pitressin®. After the flow of urine had reached 15 cc. per min. urine was collected every ten minutes for 5 to 11 separate determinations of $T_m{}^cH_2O$ (9) within a range of urine flows between 15 to 30 cc. per min. In any one experiment, throughout the range of urine flows studied, individual determinations of $T_m{}^cH_2O$ did not vary more than 0.5 cc. per min. from the mean. Venous blood was collected from the arm at appropriate intervals through an indwelling needle. Osmolality of serum and urine was determined with a Fiske osmometer. The quantity of solute-free water ($T_m{}^cH_2O$) required to restore the urine to an isosmotic state with respect to the glomerular filtrate was calculated as the difference between osmolar clearance and urine flow. Serum sodium was measured with a flame photometer. Clearances of urea (10) and inulin (11) were also measured. Data were analyzed with "Student's" "t" test.

## RESULTS

### *General*

Throughout the three days of dehydration subjects experienced moderate thirst, particularly marked after eating. Salivary flow, however, was not grossly diminished and although the tongue and mouth felt dry, they did not look dry. An increase in fatigue and irritability and loss of mental

[1] Supported by grants from the U. S. Public Health Service, the Wyndham County, New Haven (Conn.) and American Heart Associations and a contract (MD-116) with the Office of the Surgeon General, Department of the Army.

[2] Established Investigator of the American Heart Association.

[3] Parke, Davis and Co. Lot Number R983M.

TABLE I

*Maximum urinary concentration during Pitressin® infusion following various conditioning regimens*

| Subject and Diet | Dehydration 3 Days | | | | | Drink ad lib 3 Days | | | | | Force Fluids 3 Days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $U_{osm.}$ *mOsm./K* | V *cc./min.* | $U_{osm.}V$ *μOsm./min.* | $U_{urea}V$ *μOsm./min.* | $\frac{U_{osm.}}{P_{osm.}}$ | $U_{osm.}$ *mOsm./K* | V *cc./min.* | $U_{osm.}V$ *μOsm./min.* | $U_{urea}V$ *μOsm./min.* | $\frac{U_{osm.}}{P_{osm.}}$ | $U_{osm.}$ *mOsm./K* | V *cc./min.* | $U_{osm.}V$ *μOsm./min.* | $U_{urea}V$ *μOsm./min.* | $\frac{U_{osm.}}{P_{osm.}}$ |
| F. E. Low prot. | 1,048 | 0.28 | 294 | 74 | 3.75 | 717 | 0.53 | 376 | 118 | 2.56 | 658 | 1.02 | 670 | 88 | 2.30 |
| C. K. Low prot. | 1,000 | 0.40 | 400 | 97 | 3.37 | 858 | 0.46 | 394 | 93 | 3.03 | 617 | 0.68 | 418 | 83 | 2.21 |
| A. H. Regular | 1,296 | 0.51 | 657 | 305 | 4.32 | 1,104 | 0.83 | 836 | 283 | 4.00 | 879 | 0.63 | 555 | 198 | 3.02 |
| F. E. Regular | 1,180 | 0.47 | 555 | 310 | 3.93 | 822 | 0.95 | 780 | 326 | 2.93 | 725 | 1.63 | 1,180 | 322 | 2.52 |
| C. K. Regular | 1,204 | 0.85 | 1,050 | 152 | 4.07 | | | | | | 785 | 0.95 | 750 | 137 | 2.66 |
| M. E. Regular | 1,172 | 0.64 | 746 | 302 | 4.00 | | | | | | 823 | 0.59 | 487 | 172 | 2.86 |
| D. G. Regular | 1,374 | 0.25 | 344 | 154 | 4.48 | | | | | | 776 | 0.53 | 411 | 165 | 2.58 |
| C. K. High prot. | | | | | | 1,009 | 1.17 | 1,180 | 590 | 3.46 | 803 | 1.00 | 803 | 326 | 2.76 |
| Mean | 1,182 | 0.49 | 578 | 199 | 3.99 | 902 | 0.79 | 713 | 262 | 3.20 | 758 | 0.88 | 659 | 186 | 2.61 |
| P | <0.01 | 0.05 | >0.5 | >0.5 | <0.01 | 0.02 | 0.5 | >0.5 | >0.5 | <0.01 | <0.01 | 0.02–0.05 | >0.5 | >0.5 | <0.01 |
| | "Dehydration" vs. "Drink ad lib" | | | | | "Drink ad lib" vs. "Force Fluids" | | | | | "Force Fluids" vs. "Dehydration" | | | | |

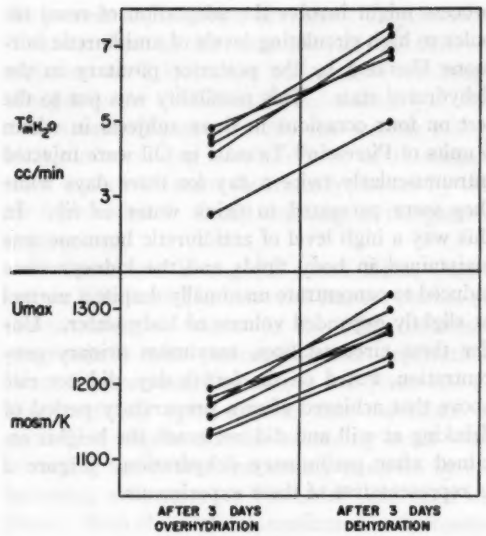


FIG. 1. EFFECT OF BODILY HYDRATION ON RENAL CONCENTRATING ABILITY

efficiency were uniformly reported. Daily output of urine varied from 350 to 700 cc. Thirst as well as fatigue were somewhat ameliorated in two subjects by 5 mg. of d-amphetamine sulfate twice a day, as suggested by Adolph (12). In contrast, while drinking 5 to 6 liters of water per day for three days, subjects reported less fatigue and no malaise, but usually developed an understandable aversion to water. One subject reported a perceptible increase in nervousness. The phenomenon of continued thirst after cessation of forced drinking (13) was not encountered.

During the infusion of hypertonic mannitol subjects frequently experienced headache when they stood up to void, which was completely relieved by straining in the Valsalva maneuver or by lying down, a sequence suggestive of lowered cerebrospinal fluid pressure.

### *Maximum urinary concentration at low flows (Table I, Figure 1)*

The solute concentration of the urine and the ratio of osmolality of urine to that of plasma achieved after maximal stimulation with Pitressin® were consistently greater in all subjects following a dehydrating regimen than after a preliminary period of forcing fluids. Moreover, dehydration raised and overhydration lowered the maximum concentration of the urine and maximum U/P ratio when compared with values achieved by the same subject after three days of drinking fluids *"ad lib."* During the three days of water restriction the osmolality of urine collected on successive days before breakfast rose progressively and was not increased further by Pitressin®. The output of total solutes and the excretion of urea, measured on the experimental

TABLE II

*Effect on $T_m{}^cH_2O$, $C_{In}$ and $C_{urea}$ of forced drinking (FD) and dehydration (D)* *

| Subject and Surface area | Regimen | $T_m{}^cH_2O$ cc./min. | $C_{In}$ cc./min. | $\frac{T_m{}^cH_2O}{C_{In}} \times 100$ | $C_{urea}$ cc./min. | $\frac{C_{urea}}{C_{In}}$ | Extremes of urine flow cc./min. | Serum Na mEq./L. | Serum osm. mOsm./K | Serum urea mM/L. |
|---|---|---|---|---|---|---|---|---|---|---|
| F. E. | FD | 4.8 | 103 | 4.7 | 80 | 78 | 20–30 | 141 | 288 | 7.3 |
| 1.8 (m.²) | D | 6.7 | 107 | 6.3 | 82 | 77 | 20–30 | 148 | 300 | 11.8 |
| R. S. | FD | 4.4 | 108 | 4.1 | 86 | 79 | 22–35 | 155 | 300 | 8.6 |
| 1.9 (m.²) | D | 6.9 | 100 | 6.9 | 79 | 80 | 17–23 | 150 | 296 | 6.7 |
| M. E. | FD | 4.6 | 98 | 4.7 | 73 | 75 | 18–29 | 151 | 287 | 4.4 |
| 1.9 (m.²) | D | 7.5 | 95 | 7.9 | 79 | 83 | 18–25 | 153 | 294 | 8.1 |
| D. G. | FD | 4.1 | 150 | 2.7 | 90 | 60 | 22–30 | 146 | 291 | 6.0 |
| 2.1 (m.²) | D | 7.3 | 135 | 5.4 | 92 | 68 | 20–27 | 148 | 293 | 8.4 |
| A. H. | FD | 2.5 | 93 | 2.7 | 75 | 81 | 15–33 | 139 | 301 | 5.2 |
| 1.7 (m.²) | D | 5.0 | 86 | 5.8 | 69 | 80 | 20–30 | 156 | 307 | 8.4 |
| Mean | FD | 4.08 | 110 | 3.78 | 81 | 75 | | 147 | 293 | 6.3 |
| | D | 6.68 | 104 | 6.46 | 80 | 78 | | 151 | 298 | 8.7 |
| P | | <0.01 | 0.1–0.2 | <0.01 | >0.5 | >0.5 | | 0.2–0.3 | 0.2–0.3 | <0.01 |

* All clearances were measured during mannitol diuresis, within the urine flows indicated.

morning during infusion of Pitressin®, were not altered consistently by preliminary water deprivation or water drinking.

*$T_m{}^cH_2O$, $C_{In}$, $C_{urea}$ (Table II, Figure 1)*

The maximum quantity of "free" water reabsorbed per minute by the kidneys during mannitol diuresis ($T_m{}^cH_2O$) (9) was strikingly altered in every subject by preliminary regimens of dehydration or overhydration. Mean $T_m{}^cH_2O$ following dehydration in five subjects was 6.7 cc. per min., averaging 65 per cent more than in the same man following a period of three days during which fluids had been forced. The effect of fluid deprivation was not eliminated by permitting one subject, F. E., to slake his thirst after the infusion of Pitressin® had been started but before mannitol was injected.

Inulin clearances measured during an infusion of 10 per cent mannitol were usually slightly lower in subjects who had been dehydrated than they were after a period of overhydration; no difference, however, was greater than 10 per cent. Although the concentration of urea in the serum was usually higher after a subject was dehydrated than after overhydration, there was no consistent difference in urea clearance measured during the infusion of hypertonic mannitol nor any significant alteration at these high flows in the ratio of urea clearance to inulin clearance (average, 0.75).

*Effect of chronic administration of Pitressin® (Figure 2)*

A possible mechanism for the augmenting effect of prolonged dehydration on the concentrating process might involve the adaptation of renal tubules to high circulating levels of antidiuretic hormone liberated by the posterior pituitary in the dehydrated state. This possibility was put to the test on four occasions in three subjects in whom 5 units of Pitressin® Tannate in Oil were injected intramuscularly twice a day for three days while they were permitted to drink water *ad lib.* In this way a high level of antidiuretic hormone was maintained in body fluids and the kidneys were induced to concentrate maximally despite a normal or slightly expanded volume of body water. Under these circumstances, maximum urinary concentration, tested on the fourth day, did not rise above that achieved after a preparatory period of drinking at will and did not reach the heights attained after preliminary dehydration. Figure 2 is representative of these experiments.

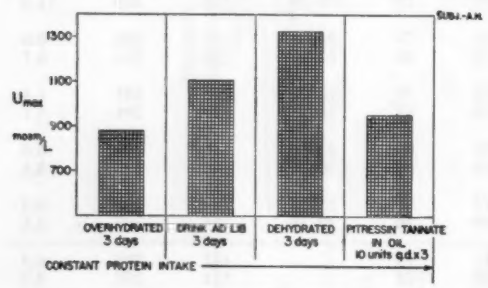


FIG. 2. EFFECT OF BODILY HYDRATION AND CHRONIC ADMINISTRATION OF PITRESSIN® ON MAXIMUM URINARY CONCENTRATION

## DISCUSSION

It seems clear that the ability of normal kidneys to concentrate the urine in response to Pitressin® may be significantly altered by a preparatory period of dehydration or of forced drinking. This effect on concentrating capacity was apparent not only at low urine flows, on maximum urinary concentration and U/P ratio, as reported by Jones and de Wardener (6) and West, Traeger, and Kaplan (4), but also on the maximum tubular reabsorptive capacity for "free" water, measured during osmotic diuresis. The magnitude of this adaptation suggests that it is of considerable physiological importance. Under conditions of prolonged dehydration, from 25 per cent to 50 per cent of urinary water losses could be conserved for bodily needs through its operation.

Several possible causes for this phenomenon are excluded or indicated by the present study. It is unlikely that differences in the amounts of circulating endogenous antidiuretic substance on the experimental day could have influenced the results, since the dose of exogenous Pitressin® injected was always well above that required to produce a maximally concentrated urine. Berliner and Davidson (14) have reported that large reductions in glomerular filtration rate produced by unilateral compression of the renal artery in dogs result in more complete reabsorption of water by the kidney whose blood supply is restricted. In

the present experiments, alterations in glomerular filtration rate in the same subject on different regimens were inconstant and minor. Although changes in the excretion of total solutes (15, 16) and of urea (7) may modify the osmotic ceiling of the urine during administration of Pitressin®, these factors were not consistently altered by water deprivation or forced drinking in the present experiments.

McCance and Young (17) and Miles, Paton, and de Wardener (5) observed that the maximum concentration of solutes in the urine increased progressively with prolonged water deprivation. West, Traeger, and Kaplan (4) and Jones and de Wardener (6) noted further that the effect of prior fluid deprivation in enhancing renal concentrating ability was not acutely reversed by rehydrating subjects after Pitressin® had been injected. Both observations, confirmed in the course of the present study, suggest that the adaptive response of the renal tubules to changes in bodily hydration is a cumulative rather than an instantaneous one, with a certain lag recalling the adaptive behavior of the kidneys with respect to the excretion of ammonia, when acid is given over several days.

The improved response of the kidneys after several days of water restriction is apparently an adjustment to something other than prolonged exposure to antidiuretic hormone. Concentrating ability was not augmented when the kidneys were forced to excrete a concentrated urine continuously for three days by injections of Pitressin® Tannate in Oil. It would appear that the renal concentrating process is conditioned by the state of hydration of body tissues (including, and perhaps most important, the cells of the distal and collecting tubules themselves). Evidence is lacking at the present time to say whether this influence is exerted at the site of action of Pitressin® or upon a separate concentrating mechanism (9).

One might predict from the results of these studies that severe reversible functional impairment of water reabsorption, resembling diabetes insipidus, would result from prolonged overdrinking. This may be the explanation of Kunstmann's report (13) of continuing thirst following a period of 8 to 12 days of over-drinking. One of us (C. K.) has recently observed a patient with psychogenic polydipsia of 15 years' duration, unable to concentrate the urine to more than 300 mOsm. per K, who gradually regained the ability to concentrate normally in response to Pitressin® after several weeks during which a normal intake of fluid was imposed.

## SUMMARY

1. The contrasting effects of water deprivation and forced drinking upon renal concentrating ability were studied in normal subjects.

2. Preliminary water deprivation increased and forced drinking decreased the maximum urinary concentration achieved after Pitressin®.

3. $T_m{}^cH_2O$ was 40 to 100 per cent higher in the same subject after dehydration than after overhydration.

4. Changes in glomerular filtration rate, total solute excretion and the excretion of urea were small and/or inconsistent.

5. Chronic administration of Pitressin® Tannate in Oil did not increase maximum urinary concentration.

6. It is suggested that the renal concentrating process is conditioned to an important degree by the state of hydration of body tissues, including those of the kidneys.

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# THE EFFECT OF FEEDING PROTEIN AND UREA ON THE RENAL CONCENTRATING PROCESS [1]

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(Submitted for publication December 7, 1956; accepted December 28, 1956)

The role of dietary protein in the treatment of renal disease remains a controversial one, in part because the influence of diets high or low in protein upon normal renal function is not completely understood. In the rat much evidence has accumulated that high-protein intakes lead to renal hypertrophy (1–3) and that low-protein diets may be associated with impairment of some renal functions (4). Pullman, Alving, Dern, and Landowne (5) have recently reviewed this subject and demonstrated that in normal men a high-protein diet slightly increases and a low-protein diet slightly depresses glomerular filtration rate, renal plasma flow and $T_m$PAH.

The present experiments were undertaken to investigate the effects of alterations in dietary protein on the ability of normal kidneys to concentrate the urine. Maximum renal concentrating capacity was altered strikingly by changing the dietary intake of nitrogen, both as protein and (an unexpected finding) as preformed urea.

## METHODS

Normal young men abstained from fluids for 12 hours prior to coming to the laboratory at 8 a.m. on the day of the experiment. In order to assure a constant and maximal antidiuretic stimulus throughout all experiments, Pitressin® [4] was infused at the rate of 200 milliunits in 50 cc. of normal saline per hour. Subjects remained in the supine position, standing only to void. Urine was collected at intervals until its osmolal concentration (freezing point) had reached a constant maximum. In some subjects mannitol was then infused, so that the maximum ability of renal tubules to reabsorb water free of solute ($T_m{}^cH_2O$) could be measured in 5 to 16 separate determinations within a range of urine flows from 10 to 35 cc. per min. Freezing points of serum and urine were determined using the Fiske osmometer. Clearances of inulin (6), creatinine (7), urea (8), sodium and potassium (9) were also determined. The "t" test of "Student" was used in analyzing the data.

Each subject was studied after three days on a *Low-Protein* diet, containing approximately 20 grams of protein per day, and a *High-Protein* diet, containing 150 to 200 grams of protein per day. The latter was achieved by the use of 100 to 200 grams per day of a high-protein, low-sodium supplement.[5] Both diets were approximately isocaloric; in some subjects the low-protein diet consisted chiefly of carbohydrate, in others fat predominated. Two subjects were studied on a *Regular* diet, containing about 90 grams of protein per day. Other variations in dietary regimen will be described under Results.

## RESULTS (Table I)

### *Effect of low- vs. high-protein diets on renal concentrating ability, inulin clearance and urea clearance (Figure 1)*

Maximum urinary solute concentration and maximum osmolal U/P ratio were invariably higher after three days of a high-protein intake than after a diet low or normal with respect to protein. The increases in maximum urinary osmolality with ingestion of large amounts of protein varied from 87 to 332 mOsm. per K. This occurred in association with an increased basal urinary flow containing from 1.2 to 5 times the quantity of solutes excreted on a low-protein diet. The maximum capacity of the kidneys to reabsorb water free of solute ($T_m{}^cH_2O$) was likewise increased by 15 to 50 per cent in five subjects by feeding protein.

Inulin clearance was slightly higher in each of five subjects on a high-protein regimen than on a diet low in protein. At the high urine flows obtained during mannitol diuresis, the clearance of

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[2] Established Investigator of the American Heart Association.

[3] James Hudson Brown Student Fellow, 1955.

[4] Parke, Davis and Co., Lot No. R983M.

[5] Melactin, supplied by E. R. Squibb and Co.

TABLE I

*Effect of prior feeding with protein or urea on renal concentrating ability during Pitressin® infusion*

| Subject and Surface area | Regimen | Before infusion of mannitol | | | | | During infusion of mannitol | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $U_{max}$ *mOsm./K* | V *cc./min.* | $\frac{U_{osm.}}{P_{osm.}}$ | $U_{osm}V$ *μOsm./min.* | $U_{urea}V$ *μOsm./min.* | $T_m{}^cH_2O$ *cc./min.* | $C_{In}$ *cc./min.* | $\frac{T_m{}^cH_2O}{C_{In}}\times 100$ | $\frac{C_{urea}}{C_{In}}\times 100$ |
| F. E. | Low prot. | 717 | 0.53 | 2.56 | 376 | 118 | | | | |
| 1.8 (m.²) | High prot. | 1,049 | 1.08 | 4.10 | 1,134 | 367 | | | | |
| | Low prot. Force fluids. | 658 | 1.02 | 2.30 | 670 | 88 | | | | |
| | High prot. Force fluids. | 812 | 0.97 | 2.80 | 790 | 241 | | | | |
| | Regular. Force fluids. | 725 | 1.63 | 2.50 | 1,180 | 322 | 4.8 | 103 | 4.6 | 78 |
| | Reg. + 48 gm. urea q.d. Force fluids. | 812 | 2.12 | 2.70 | 1,720 | 616 | 5.5 | 110 | 5.1 | 77 |
| C. K. | Low prot. | 787 | 0.34 | 2.85 | 267 | 61 | | | | |
| 1.8 (m.²) | High prot. | 1,012 | 0.87 | 3.57 | 880 | 388 | | | | |
| | Low prot. Force fluids. | 617 | 0.68 | 2.20 | 418 | 83 | 4.0 | 85 | 4.7 | 81 |
| | High prot. Force fluids | 803 | 1.00 | 2.77 | 803 | 318 | 6.5 | 96 | 6.8 | 72 |
| | Regular. Force fluids. | 785 | 0.96 | 2.66 | 750 | 137 | | | | |
| | Low prot. + 48 gm. urea q.d. Force fluids. | 893 | 1.00 | 3.02 | 893 | 326 | 5.7 | 87 | 6.5 | 79 |
| T. T. | Low prot. | 819 | 0.34 | 2.95 | 278 | 110 | 6.1 | 102 | 6.1 | 72 |
| 2.2 (m.²) | High prot. | 1,020 | 1.27 | 3.50 | 1,295 | 382 | 9.0 | 116 | 7.8 | 68 |
| McD. | Low prot. | 813 | 0.34 | 2.82 | 276 | 97 | 6.2 | 110 | 5.6 | 45 |
| 2.0 (m.²) | High prot. | 926 | 1.27 | 3.24 | 666 | 295 | 7.9 | 127 | 6.2 | 42 |
| C. H. | Low prot. | 895 | 0.42 | 3.14 | 376 | 118 | 4.6 | 112 | 4.1 | 62 |
| 1.8 (m.²) | High prot. | 1,014 | 0.72 | 3.48 | 710 | 365 | 7.5 | 118 | 6.4 | 70 |
| R. H. | Low prot. | 836 | 0.16 | 3.06 | 132 | 31 | 6.2 | 121 | 5.1 | 71 |
| 1.9 (m.²) | High prot. | 969 | 0.78 | 3.23 | 760 | 284 | 7.6 | 123 | 6.2 | 76 |
| | Low prot. + 48 gm. urea q.d. | 1,046 | 0.50 | 3.49 | 523 | 321 | 8.2 | 124 | 6.6 | 75 |
| C. K. | Low prot. | 858 | 0.46 | 3.02 | 394 | 93 | | | | |
| 1.8 (m.²) | Low prot. + 48 gm. urea q.d. | 1,009 | 1.17 | 3.46 | 1,180 | 590 | | | | |
| M. H. | Low prot. | 812 | 0.37 | 2.82 | 300 | 73 | | | | |
| 1.8 (m.²) | Low prot. + 48 gm. urea q.d. | 1,118 | 1.03 | 3.81 | 1,152 | 519 | | | | |
| C. R. | Low prot. | 760 | 0.60 | 2.68 | 455 | 163 | 5.3 | 117 | 4.5 | 68 |
| 1.8 (m.²) | Low prot. + 48 gm. urea q.d. | 929 | 1.38 | 3.28 | 1,280 | 596 | 7.1 | 116 | 6.1 | 68 |
| Mean | Low prot. | 775 | 0.60 | 2.74 | 452 | 115 | 5.3 | 107 | 5.0 | 68 |
| | High prot. or high urea. | 958 | 1.08 | 3.32 | 985 | 401 | 7.2 | 113 | 6.4 | 70 |
| P | | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.2–0.3 | <0.01 | >0.5 |

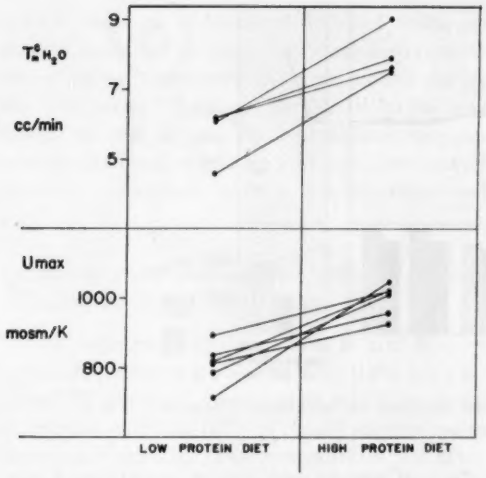


FIG. 1. EFFECT OF DIETARY PROTEIN ON RENAL CONCENTRATING ABILITY

urea averaged 70 per cent of inulin clearance. Protein feeding did not change this ratio.

*Failure of sodium chloride to improve renal concentrating ability (Figure 2)*

In contrast to the effects of protein ingestion on renal concentrating ability, maximum urinary concentration, maximum U/P ratio and $T_m{}^cH_2O$ were unchanged or slightly diminished when a low-protein diet was supplemented by 20 grams of sodium chloride per day (equivalent in osmolar terms to the urea from 100 grams of protein) for three days prior to the test. An increased excretory burden of solute supplied as electrolyte did not, therefore, produce the same increase in renal concentrating capacity as did solute derived from protein.

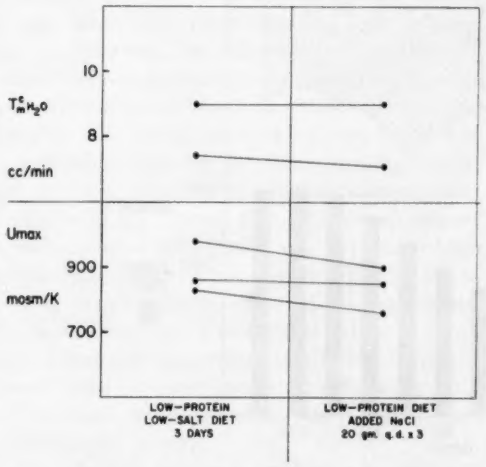


FIG. 2. EFFECT OF NaCl ON RENAL CONCENTRATING ABILITY

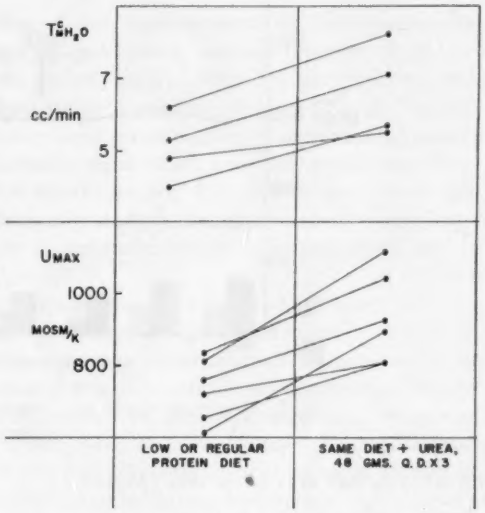


FIG. 3. EFFECT OF UREA ON RENAL CONCENTRATING ABILITY

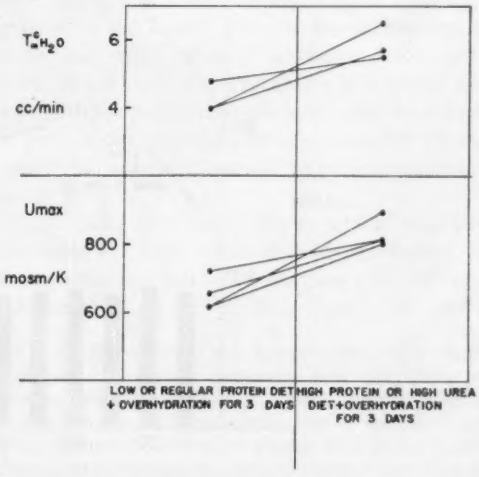


FIG. 4. EFFECT OF PROTEIN AND UREA ON RENAL CONCENTRATING ABILITY IN OVERHYDRATED SUBJECTS

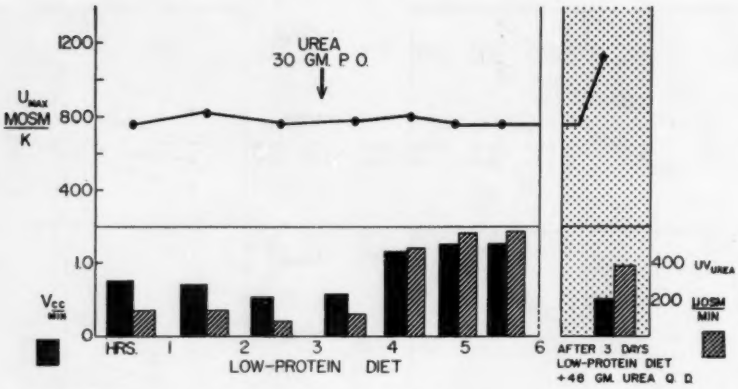


FIG. 5. EFFECT OF ACUTE VS. CHRONIC UREA LOADING ON MAXIMUM URINARY CONCENTRATION

*Effect of adding urea to the diet (Figure 3)*

When a low-protein diet was supplemented for three days, not by 140 grams of extra protein but by an equivalent amount of nitrogen in the form of 48 grams of urea daily, a marked increase occurred in maximum urinary concentration, maximum U/P ratio and $T_m{}^cH_2O$. This effect, observed in six instances, was in all respects indistinguishable from that seen in subjects eating a diet high in protein.

*Effects of protein and urea in overhydrated subjects (Figure 4)*

It seemed possible that the effects of protein and urea might be secondary to some slight or inapparent dehydration consequent to solute diuresis and insufficiently compensated for by the subject's sense of thirst. Two subjects (F. E. and C. K.) were studied repeatedly on diets low and high in protein, with and without supplements of urea, taken during a period of three days during which

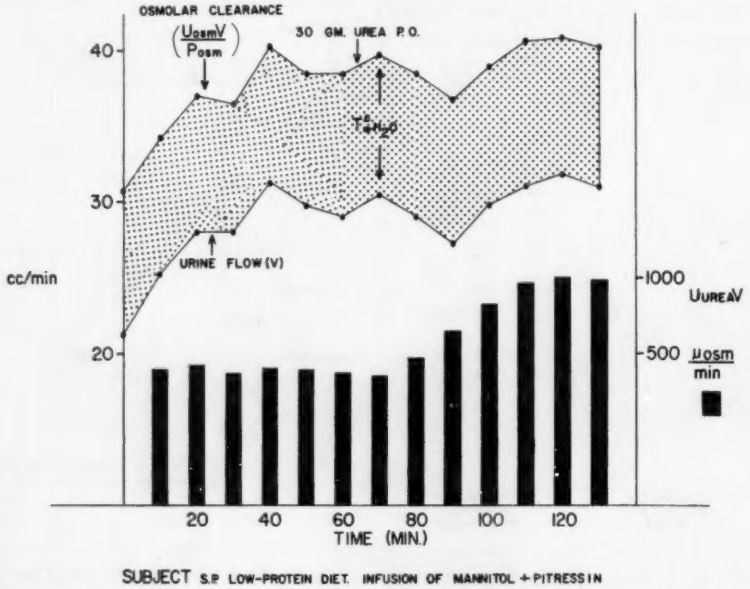


FIG. 6. FAILURE OF ACUTE INGESTION OF UREA TO INCREASE $T_m{}^cH_2O$

5 to 6 liters of water were imbibed daily, an amount sufficient to keep the measured concentration of each specimen of urine voided during this time below 150 mOsm. per K. With the possibility of dehydration thus eliminated, supplementary feedings of protein and urea consistently increased maximum urinary concentration and $T_m{}^cH_2O$.

*Failure of acute loading with urea to increase $U_{max}$ or $T_m{}^cH_2O$ (Figures 5 and 6)*

Four subjects who had eaten a diet low or normal in protein for the preceding three days ingested 30 grams of urea acutely while receiving an infusion of Pitressin®. A typical experiment is shown in Figure 5. The osmolality of the urine was essentially unaltered although the excretion of urea quadrupled. On the other hand, maximum urinary concentration was considerably increased in every case after the same subject supplemented his diet with urea for three days. $T_m{}^cH_2O$ was not changed by the acute ingestion of 30 grams of urea in two subjects in whom this was tested (Figure 6).

## DISCUSSION

Data in the literature concerning the influence of dietary variations on renal concentrating ability are scanty and conflicting. Addis and Shevky (10) suggested that healthy persons deprived of water might excrete urine of subnormal specific gravity when their diets contained little salt and protein; however, the differences which they obtained were of questionable statistical significance and were not confirmed by Miller, Price, and Longley (11) or by Addis and Foster (12), who in addition pointed out the fallacy of using specific gravity as a measure of osmolar concentration. McCance (13) found that maximum urinary osmolarity after dehydration was not changed appreciably by omitting salt from the diet or by ingesting urea on the morning of the experiment. Hayman, Shumway, Dumke, and Miller (14) reported that two dogs when deprived of fluids excreted urine of lower specific gravity while on a cracker-meal diet than when eating a diet composed largely of meat.

The effect of protein in producing hypertrophy of the kidneys of rats is well known (1–3). When the diet of rats is supplemented by nitrogen in the form of urea, renal hypertrophy, less marked than with protein feeding, has been reported (2, 15, 16) and denied (1, 3). Other urinary solutes, with the possible exception of phosphate, do not enhance renal growth (1). Protein feeding greatly increases renal blood flow and glomerular filtration rate in the dog (17) but in man (5) it produces only a slight, though statistically significant, rise in the clearances of inulin and PAH and in $T_m$PAH.

The idea that urea might obligate less water in the urine than an equimolar quantity of salt, or that maximum concentrations of urea could be attained in a urine already maximally concentrated with respect to salt was suggested by earlier workers (18, 19), but Hervey, McCance, and Taylor (20, 21) concluded from acute studies in healthy persons during hydropenia that at every urine flow there was a limiting osmotic pressure which did not discriminate between osmols of urea and osmols of salt.[6] The present experiments indicate that in order for urea to raise maximum urinary osmolarity it must be administered over several days. It is therefore unlikely that its effect can be explained solely or chiefly by an unusual ability of the renal tubules to concentrate urea *per se* to a greater extent than other solutes. If that were the case, prior feeding of protein or urea would not increase urinary osmolality measured during the determination of $T_m{}^cH_2O$, when mannitol and not urea is the chief osmotic constituent of the urine. Even if the assumption is made that urea obligates no water at all, only 20 to 50 per cent of the increases in $T_m{}^cH_2O$ observed in the present experiments after diets high in protein or urea could be accounted for by the presence of additional urea in the urine under mannitol diuresis.

The ability of renal tubules to secrete urea into the urine has been established for the kidney of the aglomerular fish (22), the frog (23, 24) and the kangaroo rat (25). It is conceivable that a

[6] It should be pointed out, however, that there are no published data dealing intensively with the effects of urea vs. other solutes on the limiting osmotic pressure of the urine in man below a urine flow of about 3 cc. per min. Scattered observations suggest that during hydropenia a maximum urinary osmolal concentration may be maintained at somewhat higher levels of urine flow and solute excretion when urea is utilized as a loading solute than when sodium chloride is loaded (13).

similar mechanism, stimulated by the necessity to excrete large amounts of urea, might operate in humans to add urea to tubular urine at or past the point where water is abstracted in the final concentrating process. Such a process should, however, be reflected in a rise in the urea/inulin clearance ratio. This did not occur in the present experiments in which $C_{urea}/C_{inulin}$, measured during osmotic diuresis, was remarkably constant on both high and low-protein diets. Secretion of urea by renal tubules is therefore not a likely explanation for the augmentation of renal concentrating ability which was observed during mannitol diuresis in persons fed protein or urea. These data do not rule out the possibility that increased tubular secretion of urea might operate at low urine flows to increase maximum urinary osmolality, an effect which might be submerged in the flood of proximal tubular urine accompanying mannitol diuresis. It is interesting in this connection that Schmidt-Nielsen observed that mannitol appeared to block tubular excretion of urea in the kangaroo rat (25).

Although the kidneys can apparently be "trained" to concentrate more or less efficiently by several days of water deprivation or of forced drinking (26), the explanation of the influence of protein and urea on the concentrating process does not lie with any tendency they might have to produce dehydration. Dietary supplements of protein and urea improved renal concentrating ability in continuously overhydrated subjects as well as in persons permitted to drink at will.

It seems clear that physiological variations in dietary protein and urea excretion, as well as in the state of bodily hydration (26), produce well-marked and separable adaptive responses on the part of the renal tubules which have an obvious utility in terms of the body's economy of water. Such effects upon renal concentrating ability cannot be neglected in future considerations of the effects of disease or therapy upon this important function of the kidneys.

## SUMMARY

1. Changes in the dietary intake of nitrogen, either as protein or urea, over a period of three days, in normal subjects produced well-marked parallel changes in maximum urinary osmolal concentration and in $T_m{}^cH_2O$.

2. Feeding of protein or urea increased the renal response to Pitressin® in continuously overhydrated subjects as well as in persons permitted to drink at will.

3. Although $U_{max}$ and $T_m{}^cH_2O$ were increased by chronic administration of urea, they were not increased by acute urea loading.

4. The data suggest that both protein and urea, when administered chronically, promote an adaptive response by the renal tubules by which water is conserved more efficiently and renal concentrating ability is augmented.

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# ETHANOL FRACTIONATION OF PLASMA AND ELECTROPHORETIC IDENTIFICATION OF INSULIN-BINDING ANTIBODY

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In previous communications (1, 2) the presence of an insulin-binding globulin in the serum of insulin-treated patients was demonstrated, *in vitro*, by paper and starch electrophoresis and by ultracentrifugal fractionation employing insulin-$I^{131}$ as a tracer. These findings were correlated with a slower disappearance of insulin-$I^{131}$ from the blood stream of insulin-treated subjects than of control subjects. Insulin-binding globulin could be demonstrated only in insulin-treated subjects (diabetic or non-diabetic) but not in subjects who had never received insulin (control subjects). It appeared within four and one-half months after institution of insulin therapy in a newly treated subject under observation. It was concluded therefore that insulin-binding globulin satisfied the criteria for antibody (1, 2). The insulin-antibody complex appeared to migrate with the fast moving $\gamma$ globulins or just in advance of the $\gamma$ globulins on starch block and paper electrophoresis (1, 2) and preliminary experiments with ethanol fractionation of the serum suggested further that the antibody might reside in the $\beta$ rather than in the $\gamma$ globulin fraction (2). However, Weiger and Colwell (3) and Peters, Burrows, and Lowell (4) have since reported that, on paper electrophoresis, insulin-$I^{131}$ migrates with the $\gamma$ globulins in the sera of insulin-resistant patients. In view of the reports of others that insulin-neutralizing activity present in the sera of insulin-resistant patients is restricted to the $\gamma$ globulin fraction (5–7) and that an anti-insulin factor present in the sera of patients with diabetic acidosis is associated with the $\alpha$ and $\beta$ globulins (8), further studies seemed indicated.

## METHODS

Nine insulin-treated diabetic patients at the Bronx Veterans Administration Hospital served as subjects for this study. They had been under insulin therapy for at least four months and were in good control. None had ever experienced "insulin resistance." ACD plasma or serum obtained at least 24 hours after the last dose of insulin from each of these patients was fractionated with cold ethanol according to the methods of Lever and associates (9) into the following 3 fractions: II, I + III, IV − VI. Table I summarizes the distribution of electrophoretic components in the various fractions according to these authors (9). Since fraction VI contains only 2.2 per cent of the total plasma proteins, further separation of fraction IV − VI into fraction IV + V and fraction VI was not undertaken. In some cases where ACD plasma rather than serum was used, fibrinogen was partly precipitated from fraction I + III during prolonged dialysis and was removed subsequently. There was never any significant precipitation of insulin-$I^{131}$. In some cases all fractions were concentrated to a volume of

TABLE I

*Distribution of total protein and electrophoretic components in plasma protein fractions [from Lever and associates (9)]*

| % Total plasma proteins | Fraction II 10.8 | I + III 22.0 | IV + V 65.6 | VI 2.2 |
|---|---|---|---|---|
| | % of fraction | | | |
| Albumin | 3.1 | 0.5 | 81.3 | |
| $\alpha_1$ | 0.1 | 3.5 | 7.8 | |
| $\alpha_2$ | | 13.3 | 4.7 | |
| $\beta_1$ | | 49.4 | 5.5 | |
| $\beta_2$ | 3.8 | 26.5 | 0.7 | |
| Fibrinogen | | | | |
| $\gamma$ | 93.0 | 6.8 | | |
| Total | 100. | 100. | 100. | |

2 ml. by freeze drying and were then dialyzed against 0.25 per cent NaCl or 0.067 M phosphate buffer, pH 7.4.

Insulin-$I^{131}$ was added in equal amounts to whole serum or plasma prior to fractionation and/or to the various fractions after separation. The mixtures were incubated at 37° for about 30 minutes and were then subjected to electrophoresis on paper. In the concentrations employed, unbound insulin-$I^{131}$ is adsorbed to the paper at the site of application ("origin") (10, 1, 2) but insulin-$I^{131}$ which is bound to any of the proteins migrates away from the origin along with the binding protein.

The insulin-$I^{131}$ employed in this investigation was prepared from crystalline beef insulin [1] with a specific activity of 3 to 12 mc. $I^{131}$ per mg. insulin and contained less than 1.0 iodine atom per 36,000 M.W. insulin. The

[1] We are indebted to Dr. O. K. Behrens and Dr. C. W. Pettinga of Eli Lilly Co. for a generous supply of crystalline regular insulin lot No. 535664.

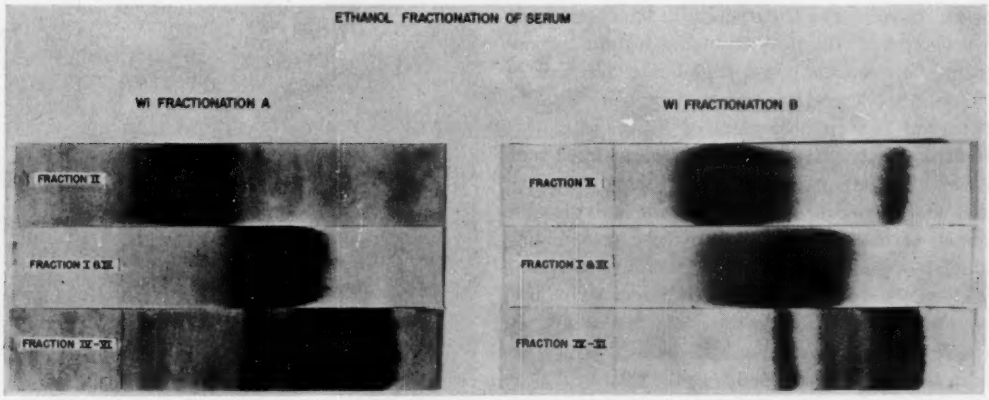


FIG. 1. PAPER ELECTROPHORESIS OF PROTEIN FRACTIONS SEPARATED BY COLD ETHANOL ACCORDING TO METHOD OF LEVER AND ASSOCIATES (9)

Two separate fractionations of same serum of subject Wi.

method of preparation has been described previously (11). In order to protect against radiation damage (2, 12) at this high specific activity, the insulin was permitted to remain in contact with the iodinating solution for only about 5 minutes or less and was then dialyzed, with continuous mixing, for 45 to 60 minutes at 4° C, against a large volume (2 liters) of distilled water. This usually removed about 90 per cent of the nonprotein bound $I^{131}$. Serum albumin was then added to a final concentration of 50 to 100 mg. per ml. to protect against further radiation damage (12) and dialysis was continued for another 7 to 18 hours until nonprotein bound $I^{131}$ was less than 1 per cent of protein bound $I^{131}$ as determined by analysis with paper electrophoresis. There was no iodination of the added serum albumin under these conditions. Since unprotected insulin was exposed to high concentrations of $I^{131}$ for less than an hour, the radiation damage incurred was minimal and did not exceed 3 to 5 per cent in any of the preparations.

## RESULTS

Although a quantitative analysis was not performed, the protein fractions generally revealed, by inspection, essentially the same gross distribution of electrophoretic components on paper electrophoresis (Fractionation A, Figure 1) as anticipated from the quantitative analysis of Lever and associates (9). In some runs, however, the separations were not as clean, gamma globulin appearing in fraction I + III or $\beta$ globulins and albumin appearing in fraction II (Fractionation B, Figure 1).

The recovery of antibody in specific fractions, as evidenced by binding on paper electrophoresis, revealed certain consistencies among the antisera studied. With two exceptions (Table II, Fo, Fractionation A and Ru) where a slight degree of binding was observed in fraction IV–VI, in addition to more marked binding in the other fractions, antibody in significant amounts was restricted to fraction I + III alone or to fraction I + III and fraction II. When antibody was present in both II and I + III, another fractionation on a sample of the same plasma revealed its presence only in fraction I + III in several cases (Table II). It was also observed that antibody was more likely to be recovered in fraction II when separation between II and I + III was incomplete, fraction II being contaminated with some of the $\beta$ globulins (*e.g.*, Subject Wi, Figure 1, Table I). In all

TABLE II

*Binding of insulin-$I^{131}$ to proteins in serum fractions separated with cold ethanol*

| Subject | Protein fraction | | |
|---|---|---|---|
| | I + III | II | IV–VI |
| Ju | + | + | 0 |
| Ru | + | + | Slight |
| Wi fractionation A | + | 0 | 0 |
| fractionation B | + | + | 0 |
| Br fractionation A | + | + | 0 |
| fractionation B | + | 0 | 0 |
| Fo fractionation A | + | + | Slight |
| fractionation B | + | 0 | 0 |
| Su | + | 0 | 0 |
| Co | + | 0 | 0 |
| Gr | + | 0 | 0 |
| La | + | 0 | 0 |

cases, regardless of the fraction in which antibody was recovered, the peak of bound insulin-$I^{131}$ migrated between the $\gamma$ and $\beta$ globulins (Figure 2) or with the slowest moving $\beta$ globulins. This localization is well demonstrated in whole plasma or serum when the paper strips are scanned with a narrow window, employing a short resolving time of the circuit, so that the radioactivities are almost precisely juxtaposed to the corresponding stained proteins (Figure 3). Confirmation was readily obtained by section of the paper strips into thin segments which were assayed individually in a well scintillation counter; the peak of bound insulin activity corresponded with the relatively clear zone between $\beta$ and $\gamma$ globulins.

Whether insulin-$I^{131}$ was added to whole plasma or serum prior to fractionation or to the various fractions after separation was immaterial to the distribution of antibody among the fractions (Figure 4).

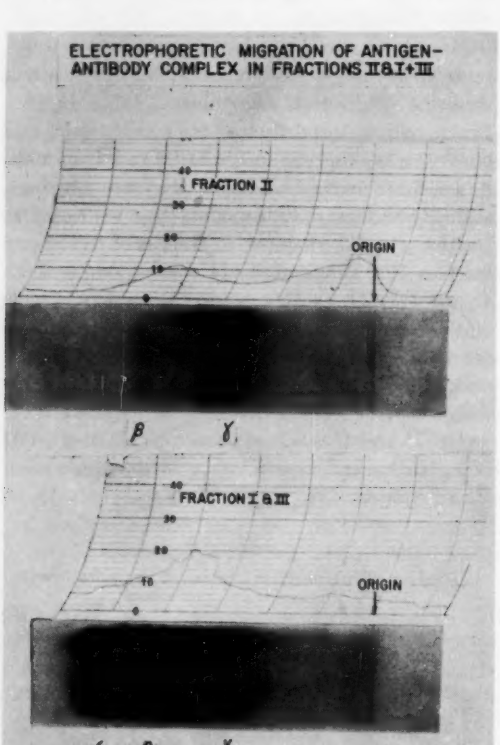


FIG. 2. PAPER RADIOELECTROPHORETOGRAMS OF FRACTIONS II AND I + III (SUBJECT JU) TO WHICH INSULIN-$I^{131}$ WAS ADDED *in vitro*

The resolving time of the recording circuit was 10 seconds so that radioactivities are about 1/6 of a vertical division to the left of the corresponding sites on the electrophoresis strips.

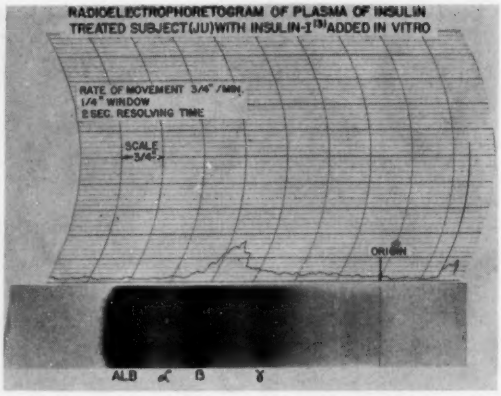
RADIOELECTROPHORETOGRAM OF PLASMA OF INSULIN TREATED SUBJECT (JU) WITH INSULIN-I(3) ADDED IN VITRO
RATE OF MOVEMENT 3/4" /MIN.
1/4" WINDOW
2 SEC. RESOLVING TIME
SCALE
ORIGIN
ALB α β γ

FIG. 3. PAPER RADIOELECTROPHORETOGRAM OF PLASMA OF SUBJECT JU TO WHICH INSULIN-$I^{131}$ WAS ADDED *in vitro*

Resolving time of recording circuit short so that radioactivity curve corresponds almost precisely to subjacent sites on the electrophoresis strip.

## DISCUSSION

The present study indicates that insulin binding antibody has an electrophoretic mobility greater than that of the main mass of $\gamma$ globulins. In whole serum or in serum fractions, the insulin-$I^{131}$-antibody complex migrated in the relatively clear zone between $\gamma$ and $\beta$ globulins. When plasma was employed, the peak of radioactivity generally corresponded to that of fibrinogen. However, the possibility that the uncomplexed insulin-binding antibody has a significantly different mobility and that its mobility is altered by binding to the insulin molecule must be considered. Since unbound insulin-$I^{131}$ migrates in the region of $\alpha_1$ globulin when adsorption to the paper is prevented (*2*), it would be expected to influence the mobility of antibody after binding in the anodal direction if at all. Bound insulin-$I^{131}$ always migrated either just in advance (towards the anode) of the stainable portion of fraction II or at the cathodal side of the proteins in fraction I + III. This position represents the line of separation between the proteins of the two fractions. Since, in the separated fractions, antibody was most frequently absent from fraction II, it gen-

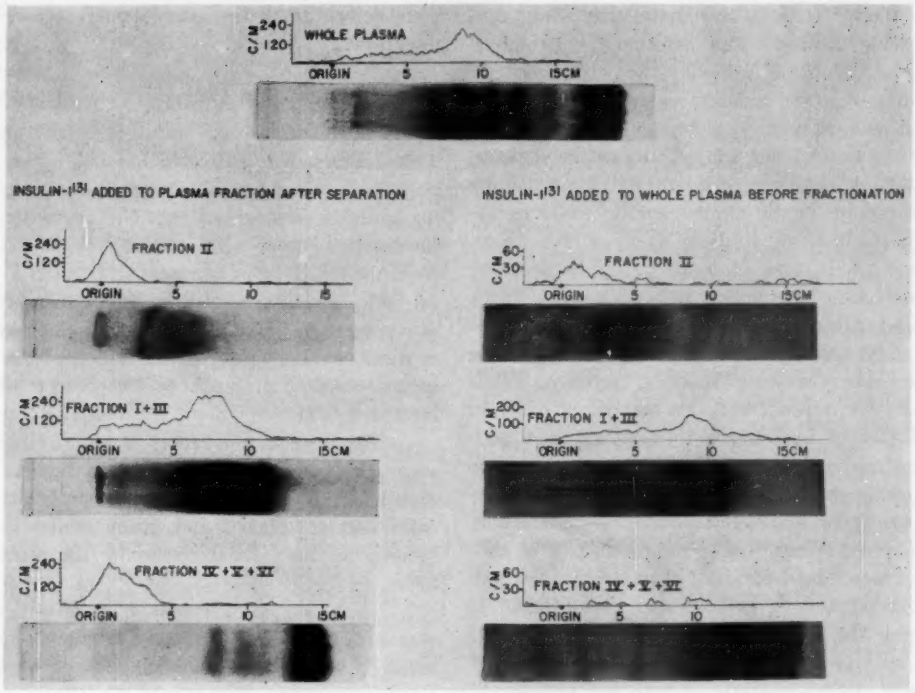


FIG. 4. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA AND PLASMA PROTEIN FRACTIONS (SUBJECT LA) TO WHICH INSULIN-$I^{131}$ HAD BEEN ADDED *in vitro* BEFORE AND AFTER FRACTIONATION WITH COLD ETHANOL

erally did not have a mobility lower than that of the slowest migrating components of fraction I + III. Since this is the zone of migration of the antibody-insulin complex, it may be inferred that binding of insulin did not affect significantly the electrophoretic mobility of insulin antibody. This observation also suggests that each antibody molecule may bind only a single insulin molecule or, at least, that the antigen-antibody complex does not contain a high ratio of insulin to antibody.

The presence of antibody in fraction II in some cases may be attributable to experimental variations in critical salt, alcohol and temperature conditions, since in these instances the fractions were not well delineated on electrophoresis and, on repeat fractionation of the same serum, antibody was frequently absent from fraction II. In any event, the insulin binding globulin migrates electrophoretically in the region of relatively low protein concentration between the $\gamma$ and $\beta$ globulins. Differentiation of this region into fastest $\gamma$ globulins or slowest $\beta$ globulins would certainly be arbitrary but the constant presence of antibody in fraction I + III rather than in fraction II (where it is present only occasionally) favors its inclusion with the $\beta$ globulins. Perhaps the best compromise is the designation "inter $\gamma$–$\beta$ region" which is, in fact, the most accurate electrophoretic characterization.

Wasserman antibody (13) and horse antipneumococcus antibody (14) are also reported to have mobilities between those of the $\gamma$ and $\beta$ globulins. It is of further interest that Cann and Loveless (15) observed reagin activity of serum in patients sensitive to cottonseed or ragweed pollen to be most closely correlated with the $\beta$ globulin content of serum protein fractions separated by electrophoresis convection, although $\gamma_1$ globulin participation could not be directly excluded. More recently, Loveless and Cann (16) have examined, by similar methods, the serum of an insulin-resistant diabetic patient who exhibited some apparent in-

sulin allergy as well, and found that while skin sensitizing antibody was associated with the $\beta$ globulins, blocking antibody was contained in fractions of lower mobility corresponding to the fastest and slowest $\gamma$ globulins or (in another specimen of the same subject) to all the $\gamma$ globulins. Their observations would indicate a definite heterogeneity in the electrophoretic characterization of the blocking antibody itself. Furthermore, because of its zone of localization, the blocking antibody would not appear to be identical with the insulin-binding antibody investigated in the present study, although the latter might, *a priori*, be suspected of possessing "blocking" activity. While it seems quite possible that a number of different antibodies to insulin are formed and that antibodies reported with different techniques are, in fact, different antibodies, it also appears reasonable to suggest that even the same antigenic stimulus may not always invoke the production of antibodies which have identical chemical compositions and electrophoretic mobilities in all subjects. In addition the possibility of distinct antibodies to pork and beef insulin should not be overlooked. In most studies mixtures of pork and beef insulin obtained from commercial sources were used in the demonstration of antibody. In the present study pure beef insulin was employed. A proper approach to the problem would appear to be the simultaneous testing of various protein fractions of the same serum for insulin-neutralizing, insulin-binding, insulin-blocking and skin sensitizing antibodies. The studies of Loveless and Cann (16) suggest that at least the last two of these activities definitely reside in different fractions. On the other hand, experiments reported elsewhere (17) indicate that the serum factor associated with the apparent inhibition of insulin degradation by liver homogenates is identical with the antibody responsible for the binding of insulin-$I^{131}$ which has been demonstrated by zone electrophoresis. It would also seem possible that insulin-binding protein and insulin-neutralizing factors may be identical antibodies. However, a recent report by Field and Stetten (8) indicates the presence of an insulin-neutralizing factor (detected by the abolishment of the insulin effect on glycogen accumulation by rat diaphragm) in diabetic acidosis which is localized to the $\alpha$ and $\beta$ globulin fractions. It is clear that considerable work remains to be done before all these findings can be completely integrated.

## SUMMARY AND CONCLUSIONS

1. The sera of nine insulin-treated subjects were fractionated with cold ethanol. The fractions were tested for the presence of insulin-binding antibody employing $I^{131}$ labeled crystalline beef insulin and paper strip electrophoresis. In all cases the antibody was present in fraction I + III ($\beta$ and $\alpha$ globulins). In five cases antibody was also present in fraction II (mainly $\gamma$ globulin) but in three of these cases refractionation of the same serum revealed antibody to be present only in fraction I + III.

2. The zone of migration of the antibody-insulin complex was in the inter $\gamma$–$\beta$ region. The distribution of antibody among the fractions indicates that complexed and uncomplexed insulin-binding antibody have essentially the same mobility.

3. There was no difference, qualitatively, in the recovery of antibody in specific fractions when insulin-$I^{131}$ was added to whole plasma or serum before or after fractionation.

## ACKNOWLEDGMENTS

We wish to thank Mr. Manuel J. Villazon for the technical assistance in performing the ethanol fractionations. We also wish to thank Miss Eve Spelke and Mrs. Frieda Steiner for secretarial assistance. We are also indebted to the Dept. of Medical Illustration for the figures.

## *Addendum*

Since this paper was submitted we have observed antibodies to pure pork insulin as well as to pure beef insulin in each of three antisera examined. In addition, marked cross reactions were observed.

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# APPARENT INHIBITION OF LIVER INSULINASE ACTIVITY BY SERUM AND SERUM FRACTIONS CONTAINING INSULIN-BINDING ANTIBODY

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A number of anti-insulin factors present in the serum of insulin-resistant subjects has been reported. Lowell (1) presented evidence for both insulin-neutralizing and insulin-sensitizing antibodies. De Fillipis and Iannaccone (2) and Colwell and Weiger (3) employing salting out methods and Sehon, Kaye, McGarry, and Rose (4) using starch electrophoresis have reported that insulin-neutralizing activity is localized in the $\gamma$ globulin fraction of the serum. Loveless and Cann (5), utilizing the technique of electrophoresis convection, observed that insulin-blocking antibody is present in the $\gamma$ globulin fraction while insulin-sensitizing antibody is recovered with the $\beta$ globulins. An anti-insulin factor, capable of inhibiting glycogen accumulation by the isolated rat diaphragm, was reported by Field and Stetten (6) to be present in the $\alpha$ and $\beta$ globulin fraction of the serum of diabetic subjects, during episodes of severe insulin-resistant acidosis.

In addition to the factors reported in cases of insulin resistance, an insulin-binding antibody has been demonstrated, by zone electrophoresis and ultracentrifugal fractionation studies employing insulin-$I^{131}$, to be present consistently in the serum of insulin treated subjects (7, 8). A study of the distribution of insulin-binding antibody among serum fractions separated with cold ethanol revealed that antibody was restricted to fraction I + III (which is comprised chiefly of $\beta$ and $\alpha$ globulins) in most subjects (9). In some, antibody was recovered in fraction II (primarily $\gamma$ globulins with a small amount of $\beta_2$ globulin) as well as in fraction I + III. In two subjects there were observed a small amount of insulin binding in fraction IV – VI (mainly albumin with a small amount of $\beta$ and $\alpha$ globulins) as well as more marked binding in the other two fractions. In all cases, bound insulin migrated in the inter $\gamma - \beta$ region on electrophoresis, regardless of the fraction in which the antibody was recovered (9).

Still another anti-insulin effect is manifested by some sera in inhibiting the degradation of insulin by rat liver homogenates. Williams (10) has recently reported that such inhibition is exhibited by normal plasma but that inhibition is much greater with diabetic than with normal sera.

The different methods employed in the demonstration of anti-insulin factors and in the fractionation of serum for their localization have made it impossible to clearly differentiate among these factors. In the present study the plasma factor associated with apparent inhibition of liver insulinase has been identified with insulin-binding antibody. Inhibitory activity was not observed in normal plasma or in plasma of diabetic subjects who had never received exogenous insulin. The apparent inhibition of liver insulinase activity described in the present report is clearly to be distinguished from that due to direct interference with the liver insulinase system. Inhibition in the present sense applies solely to the protection of insulin from the degrading action of liver insulinase as a consequence of the binding of insulin by insulin-binding antibody.

## METHODS

Insulin-$I^{131}$ was prepared from crystalline beef insulin [1] with a specific activity of 3–12 mc. $I^{131}$ per mg. insulin and protected from extensive irradiation damage (11) according to methods previously described (9). At the higher specific activities, it was not possible to prevent such damage completely and 3 to 5 per cent was frequently found to be altered. Radiation-damaged material binds more or less indiscriminately to all the proteins of control plasma (8). The extent of binding of damaged material is independent of the concentration of insulin and is identical in all control plasma samples.

Serum protein fractions II, I + III, and IV – VI were prepared according to the method of Lever and associates (12) and concentrated, by freeze-drying, to two mil-

[1] We are indebted to Dr. O. K. Behrens and Dr. C. W. Pettinga of Eli Lilly Co. for a generous supply of crystalline beef insulin Lot No. 535664.

liliter volumes before or after dialysis against 0.25 per cent NaCl and were then dialyzed against 0.067 M phosphate buffer, pH 7.4, for 18 to 24 hours.

Liver homogenates were prepared by mincing fresh pieces of rat liver with two volumes of 0.067 M phosphate buffer in a Virtis homogenizer. Following centrifugation for 5 minutes at 2,000 g, the supernatant material was employed without further dilution or diluted 1:10 with the phosphate buffer. Where necessary, pH was again adjusted to 7.4.

The extent of binding of insulin by serum antibody was determined by paper electrophoresis or hydrodynamic flow paper chromatography, employing insulin-$I^{131}$, according to methods described elsewhere (8). When insulin-$I^{131}$ is added to serum or serum protein fractions and subjected to electrophoresis or chromatography on paper, insulin which is not bound to any of the proteins is adsorbed to the paper at the site of application ("origin") (13, 7, 8) but insulin which is bound to any of the proteins migrates away from the origin along with the binding protein. Since some dissociation of the insulin-antibody complex may occur during the time required to complete electrophoresis, the presence or absence of binding in specific fractions was frequently determined by simple hydrodynamic flow chromatography (8), which effects satisfactory movement of the plasma proteins away from the origin in 20 to 30 minutes.

*Experimental procedures*

*Uninhibited reaction.* Insulin concentrations ranging from 0.2 to 500 μg. per ml. were prepared in solutions of 0.067 M phosphate buffer containing serum albumin, 25 to 50 mg. per ml., for the purpose of minimizing adsorption of insulin to the walls of glass containers (14). Tracer insulin-$I^{131}$ containing 400 millimicrograms insulin was added to each solution and was taken into account in calculation of the total insulin concentration.

Substrate solutions and preparations of liver homogenate were placed in a constant temperature water bath at 37° C and allowed to reach temperature equilibrium.

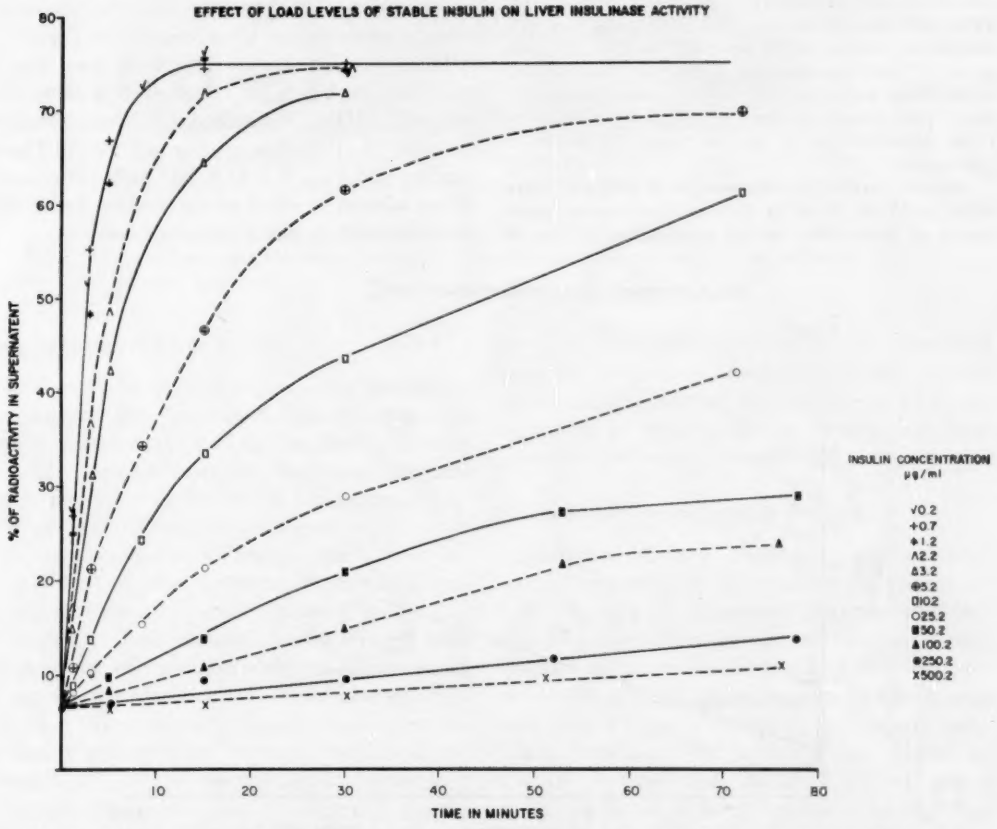


FIG. 1. DEGRADATION OF INSULIN BY RAT LIVER HOMOGENATE AT VARIOUS CONCENTRATIONS OF INSULIN
Each substrate solution contained 0.2 μg. per ml. insulin-$I^{131}$.

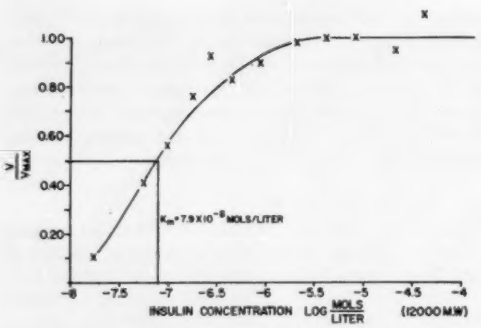


FIG. 2. INSULIN DEGRADATION ($V/V_{max}$) AS A FUNCTION OF SUBSTRATE CONCENTRATION

One milliliter of a 1 : 10 dilution of liver homogenate was added to one milliliter substrate solution and mixed instantly. At various intervals, 0.1 or 0.2-ml. aliquots were removed and pipetted into test tubes containing 5 ml. cold 10 per cent trichloracetic acid. Precipitates and filtrates were assayed separately for radioactivity in a well scintillation counter with a sensitivity of $1.00 \times 10^6$ cpm. per $\mu$c. $I^{131}$ over a background of 200 cpm. Radioactivity in the filtrate was taken to represent degraded insulin-$I^{131}$. It has been shown elsewhere (15) that degradation involves proteolytic activity and not simply the process of deiodination.

*Inhibited reaction.* Known amounts of insulin-$I^{131}$ were added to 0.5 or 1.0 ml. of dialyzed whole serum, whole plasma or plasma fraction and incubated at 37° for 30 minutes. Control solutions containing phosphate buffer, serum albumin, and insulin-$I^{131}$ as described above were run simultaneously. Aliquots of all solutions were removed for electrophoretic or chromatographic analysis. One-tenth or 0.5 ml. undiluted liver homogenate, or 1.0 ml. of a 1 : 10 dilution of liver homogenate was then added to the substrate solutions and incubation at 37° C was continued. Aliquots were taken at various intervals and added to cold 10 per cent trichloracetic acid as described above. For any individual experiment, volumes and dilutions of liver homogenate employed were identical for all substrate solutions.

## RESULTS

### *Uninhibited reaction*

The fraction of insulin-$I^{131}$ degraded as a function of time is plotted for various insulin concentrations in Figure 1. The initial velocity at each concentration was calculated from the product of the zero time slope and the initial insulin concentration and is plotted as fraction of the maximum velocity *vs.* substrate concentration in Figure 2. A Lineweaver-Burk plot (16) of the same data is presented in Figure 3. Assuming a molecular weight of 12,000 for insulin, the Michaelis-Menten constant ($K_m$) for this reaction is $7.9 \times 10^{-8}$ mols per liter and $V_{max}$ is $2.50 \times 10^{-8}$ mols per minute. When account is taken of the dilution factor this amounts to 18 $\mu$g. min.$^{-1}$ per gram liver.

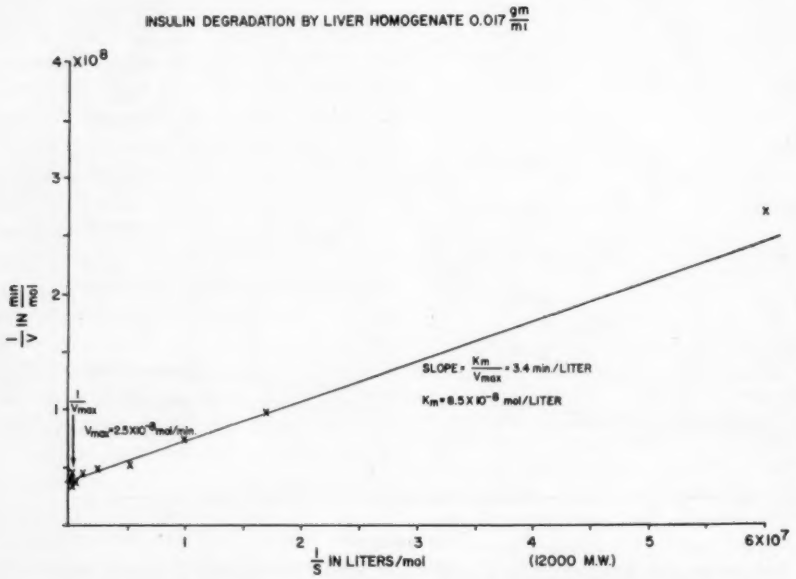


FIG. 3. LINEWEAVER-BURK PLOT OF SAME DATA AS IN FIGURE 2

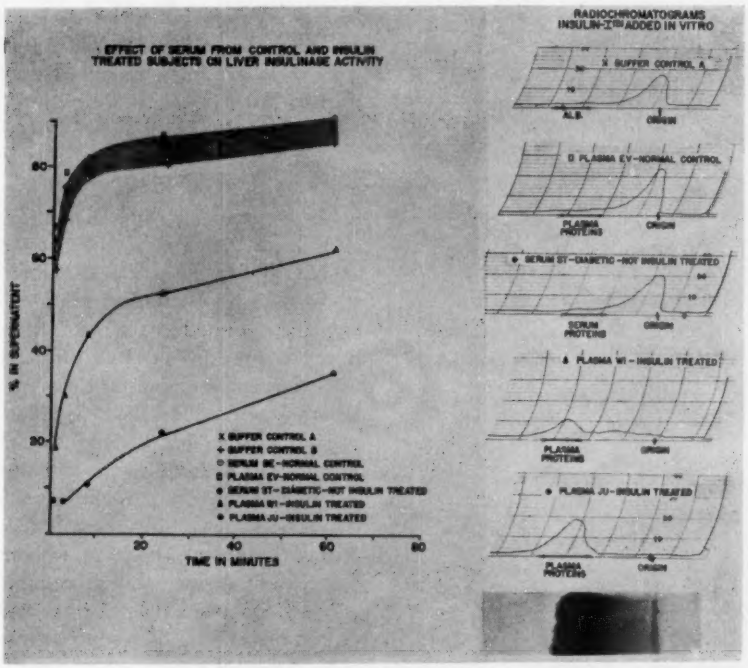
EFFECT OF SERUM FROM CONTROL AND INSULIN TREATED SUBJECTS ON LIVER INSULINASE ACTIVITY
% IN SUPERNATENT
TIME IN MINUTES
X BUFFER CONTROL A
+ BUFFER CONTROL B
○ SERUM BE-NORMAL CONTROL
□ PLASMA EV-NORMAL CONTROL
● SERUM ST-DIABETIC-NOT INSULIN TREATED
▲ PLASMA WI-INSULIN TREATED
■ PLASMA JU-INSULIN TREATED
RADIOCHROMATOGRAMS
INSULIN-I¹³¹ ADDED IN VITRO
X BUFFER CONTROL A
□ PLASMA EV-NORMAL CONTROL
● SERUM ST-DIABETIC-NOT INSULIN TREATED
▲ PLASMA WI-INSULIN TREATED
■ PLASMA JU-INSULIN TREATED
ORIGIN
PLASMA PROTEINS
SERUM PROTEINS

FIG. 4. THE EFFECT OF SERUM AND PLASMA OF INSULIN TREATED AND CONTROL SUBJECTS ON THE RATE OF DEGRADATION OF INSULIN-$I^{131}$ BY RAT LIVER HOMOGENATES

The small fraction of radioactivity migrating with albumin in the buffer control studies and with the plasma protein of control subjects represents radiation damaged material (see text).

*Reaction with serum or plasma*

The serum or plasma of subjects (diabetic or nondiabetic) who had never received exogenous insulin revealed no evidence of insulin-binding on paper electrophoresis and chromatography and had no influence on the rate of degradation of insulin-$I^{131}$ by liver insulinase (Figure 4).

In the presence of serum or plasma from insulin treated subjects, however, the rate of degradation of insulin-$I^{131}$ was inhibited significantly (Figure 4). This inhibition was correlated with the presence of insulin binding as demonstrated by paper electrophoresis or chromatography (Figure 4). When insulin concentrations were increased, resulting in an increased fraction of unbound insulin, the per cent of insulin degraded as a function of time increased, in contrast to the decrease observed in the serum of nontreated subjects (Figure 5) and in the control solutions (Figure 1). The inhibitory activity was correlated with the presence of electrophoretically demonstrable insulin binding to inter $\gamma - \beta$ globulins regardless of whether insulin therapy had been given for diabetes or schizophrenia.

*Reaction with plasma protein fractions*

Inhibition of liver insulinase activity corresponded precisely to the presence or absence of insulin binding by the specific fractions, as determined by paper electrophoresis or chromatography. In cases where insulin binding was observed only in fraction I + III, inhibition was observed only with this fraction (Figure 6). In those cases where insulin-binding antibody was present in fraction II as well as in fraction I + III (Figures 7, 8), inhibition of liver insulinase activity was evident in both fractions and was roughly proportional to the degree of binding exhibited by each

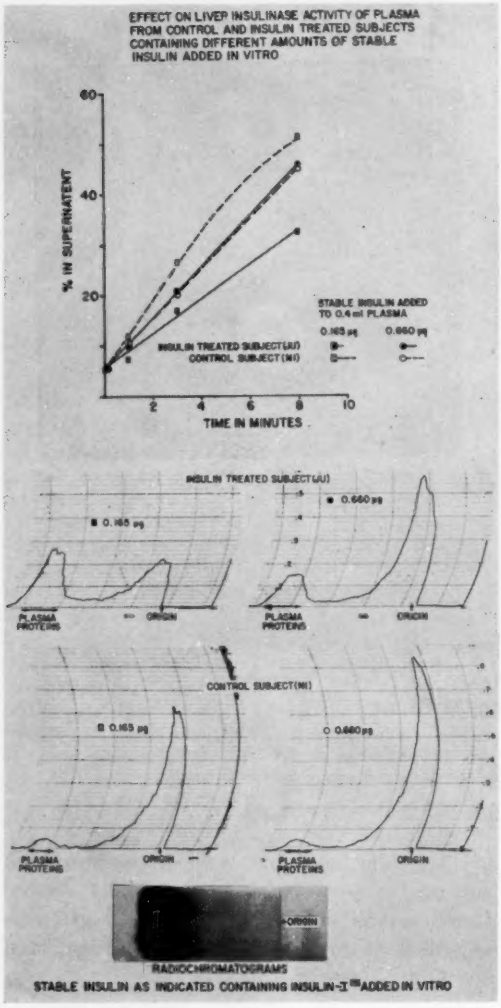
EFFECT ON LIVER INSULINASE ACTIVITY OF PLASMA FROM CONTROL AND INSULIN TREATED SUBJECTS CONTAINING DIFFERENT AMOUNTS OF STABLE INSULIN ADDED IN VITRO
% IN SUPERNATENT
TIME IN MINUTES
STABLE INSULIN ADDED TO 0.4 ml PLASMA
INSULIN TREATED SUBJECT(AU)
CONTROL SUBJECT(NI)
PLASMA PROTEINS
ORIGIN
RADIOCHROMATOGRAMS
STABLE INSULIN AS INDICATED CONTAINING INSULIN-I¹³¹ ADDED IN VITRO

FIG. 5. THE EFFECT OF VARYING SUBSTRATE CONCENTRATIONS ON THE RATE OF DEGRADATION OF INSULIN-$I^{131}$ BY RAT LIVER HOMOGENATE INCUBATED WITH PLASMA OF CONTROL AND INSULIN TREATED SUBJECTS

Note the radiation damaged material migrating with the plasma proteins of the control subject.

fraction. In one case binding was present in all three fractions and all three fractions inhibited insulin degradation by the liver homogenates.

It has been observed previously (9) that, on one fractionation, insulin-binding antibody may be present in both fraction I + III and fraction II, but on a repeat fractionation of the same serum, antibody may be recovered only in fraction I + III. In one such case studied here inhibitory activity was manifested by both II and I + III (Figure 7) when both fractions contained antibody and by fraction I + III alone when only that fraction contained antibody (Figure 6).

## DISCUSSION

Mirsky, Perisutti, and Dixon (17) have previously made a thorough study of the rat liver insulinase system. The $V_{max}$ obtained in the present study (18 $\mu$g. per min. per gram liver) is in good agreement with that (20 $\mu$g. per min. per gram liver) obtained by these workers. However, their value for $K_m$ is over 100 times as great as that reported here. This discrepancy is probably explained by the fact that Mirsky, Perisutti, and Dixon (17) determined the extent of degradation at the end of 30 minutes and assumed that degradation was linear for all substrate concentrations during this time. The present observations (Figure 1) indicate that this procedure would markedly underestimate the velocities at the lower concentrations and would lead to a gross overestimate of the value for $K_m$.

The correspondence of demonstrable insulin binding and inhibition of liver insulinase activity indicates that the two manifestations of "anti-insulin" activity are identical. This conclusion is further strengthened by the observation that an increase in insulin concentration, which reduces the ratio of bound to unbound insulin, leads to an increase in the fractional rate of degradation. This observation would be difficult to reconcile with any interpretation that inhibition of liver insulinase activity is associated with some plasma factor unrelated to insulin binding such as "insulinase inhibitor," etc., for if this were the case, increase in substrate (insulin) concentration should lead to a decrease in the fractional rate of degradation.

These studies do not confirm the observation of Williams (10) that normal plasma inhibits the insulin degrading action of liver homogenates. A possible explanation for the difference in results may be related to the addition of serum albumin to control solutions in the studies reported here. When small amounts of tracer insulin-$I^{131}$ alone are incubated with phosphate buffer and liver homogenate, significant amounts of insulin-$I^{131}$ continue to adsorb to the glass walls of the vessels during the experiment (18). Since the degraded

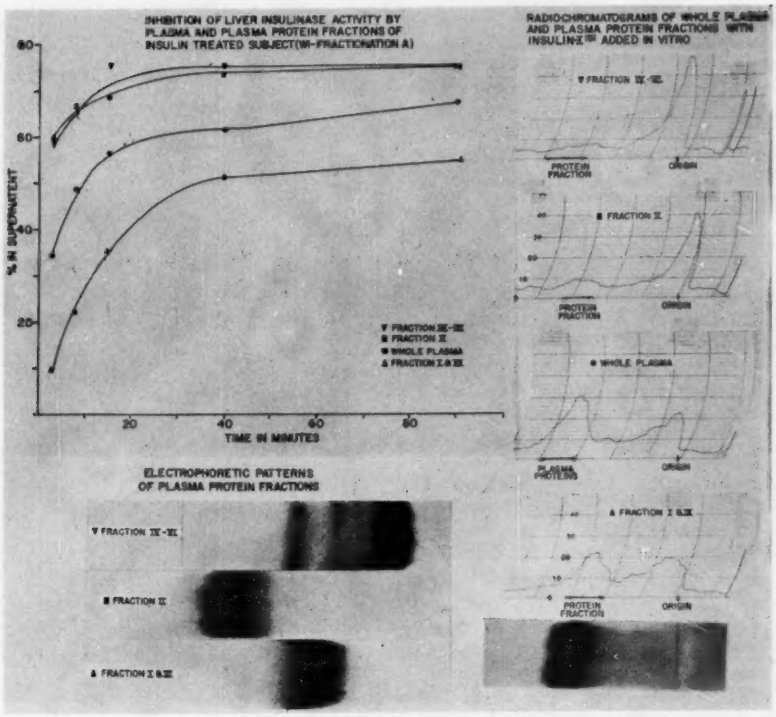
INHIBITION OF LIVER INSULINASE ACTIVITY BY PLASMA AND PLASMA PROTEIN FRACTIONS OF INSULIN TREATED SUBJECT(WI-FRACTIONATION A)
% IN SUPERNATENT
TIME IN MINUTES
RADIOCHROMATOGRAMS OF WHOLE PLASMA AND PLASMA PROTEIN FRACTIONS WITH INSULIN-I¹³¹ ADDED IN VITRO
PROTEIN FRACTION
ORIGIN
WHOLE PLASMA
ELECTROPHORETIC PATTERNS OF PLASMA PROTEIN FRACTIONS

FIG. 6. THE EFFECT OF PLASMA AND PLASMA PROTEIN FRACTIONS FROM AN INSULIN TREATED SUBJECT (WI) ON THE RATE OF DEGRADATION OF INSULIN-$I^{131}$ BY RAT LIVER HOMOGENATE AND CORRELATION WITH CHROMATOGRAPHIC EVIDENCE OF THE PRESENCE OR ABSENCE OF INSULIN BINDING

material is not adsorbed, the fraction of undegraded insulin remaining in solution at any time appears to be less than in the presence of serum or albumin, either of which serves to competitively inhibit insulin adsorption by the glass. The rate of degradation therefore *appears* to be inhibited by the presence of plasma.

Serum albumin itself has no inhibitory influence on liver insulinase activity (10, 17) nor do a number of other proteins (17). However, the competitively inhibitory effect on liver insulinase activity of certain other proteins such as glucagon (18) and the competitively inhibitory effect of insulin on liver "adrenocorticotropinase" and liver "glucagonase" activity (18) suggests that liver insulinase is not an absolutely specific enzyme system. The possibility that competitively inhibitory proteins are present in sufficiently high concentration to explain the results obtained in this study is unlikely and furthermore could not account for the increase in fractional rate of degradation with increase in insulin concentration in inhibitory sera.

The present study also fails to confirm the inhibitory influence of diabetic plasma unless the subject has received insulin therapy for some time and has produced insulin-binding antibody, since plasma of nontreated diabetic subjects contains no insulin-binding antibody and fails to inhibit insulinase activity.

The possibility of identity among some of the diverse anti-insulin activities reported by different investigators has been considered elsewhere (9). The present study indicates that at least two manifestations of "anti-insulin" activity are referable to the same phenomenon, namely binding of insulin by antibody. Whether insulin-neutralizing activity, reported to reside in the $\gamma$ globulin fraction (2–4), is identical to insulin-binding globulin, which has been shown to have an elec-

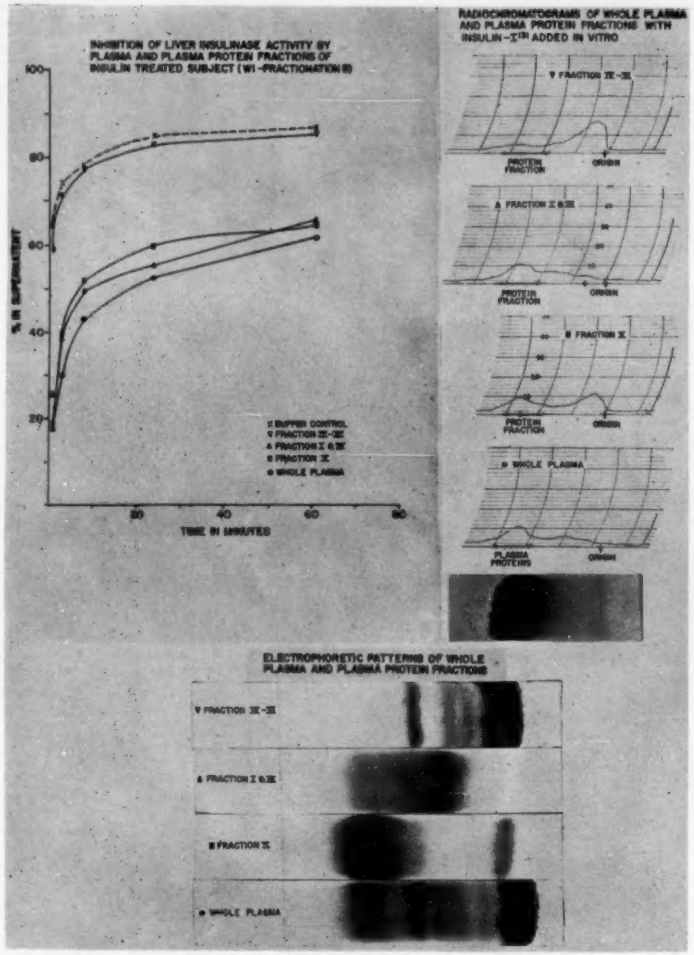
INHIBITION OF LIVER INSULINASE ACTIVITY BY PLASMA AND PLASMA PROTEIN FRACTIONS OF INSULIN TREATED SUBJECT (W1 - FRACTIONATION III)
% IN SUPERNATENT
TIME IN MINUTES
X BUFFER CONTROL
▼ FRACTION II-III
▲ FRACTION I & II
■ FRACTION I
● WHOLE PLASMA
RADIOCHROMATOGRAMS OF WHOLE PLASMA AND PLASMA PROTEIN FRACTIONS WITH INSULIN-I131 ADDED IN VITRO
PROTEIN FRACTION
ORIGIN
PLASMA PROTEINS
ELECTROPHORETIC PATTERNS OF WHOLE PLASMA AND PLASMA PROTEIN FRACTIONS

FIG. 7. SIMILAR STUDY AS IN FIGURE 6—REPEAT FRACTIONATION ON PLASMA OF SAME SUBJECT (W1)

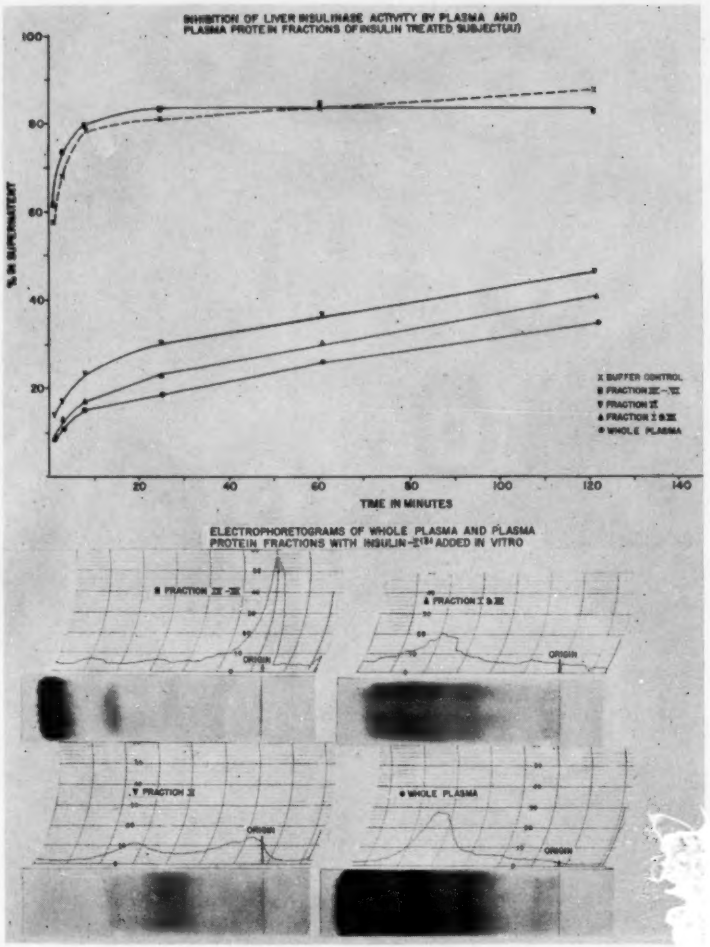
INHIBITION OF LIVER INSULINASE ACTIVITY BY PLASMA AND PLASMA PROTEIN FRACTIONS OF INSULIN TREATED SUBJECT (J1)
% IN SUPERNATENT
TIME IN MINUTES
X BUFFER CONTROL
■ FRACTION II-III
▼ FRACTION II
▲ FRACTION I & II
● WHOLE PLASMA
ELECTROPHORETOGRAMS OF WHOLE PLASMA AND PLASMA PROTEIN FRACTIONS WITH INSULIN-I131 ADDED IN VITRO
ORIGIN

FIG. 8. SIMILAR STUDY AS IN FIGURES 6, 7—SUBJECT JU

trophoretic mobility corresponding to the inter $\gamma - \beta$ region (9) cannot be answered at present. Examination of insulin-binding protein fractions for neutralizing activity appears indicated.

## SUMMARY AND CONCLUSION

1. The kinetics of insulinase activity of rat liver homogenate preparations were studied employing insulin-$I^{131}$ as a tracer. $K_m$ for the reaction was $7.9 \times 10^{-8}$ mols per liter and $V_{max}$ was found to be 18 $\mu$g. per min. per gram liver at 37° C and pH 7.4.

2. Pre-incubation of insulin-$I^{131}$ with plasma or serum of insulin-treated subjects resulted in inhibition of liver insulinase activity. No such inhibition was observed with serum or plasma of diabetic or nondiabetic subjects who had never been treated with insulin. Inhibition of liver insulinase activity was correlated with the presence of insulin-binding antibody as demonstrated by paper electrophoresis or paper chromatography.

3. When inhibitory sera were fractionated with cold ethanol, inhibitory activity of the specific fractions was correlated with the presence of insulin-binding antibody.

4. It is concluded that the apparent inhibition of liver insulinase activity by plasma or plasma fractions of insulin-treated subjects is due to the complexing of insulin by insulin-binding antibody.

## ACKNOWLEDGMENTS

We wish to thank Mr. Manuel J. Villazon for the technical assistance in performing the ethanol fractionations. We also wish to thank Miss Eve Spelke and Mrs. Frieda Steiner for secretarial assistance.

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# STUDIES ON STRUCTURE DIURETIC ACTIVITY RELATIONSHIPS OF ORGANIC COMPOUNDS OF MERCURY [1]

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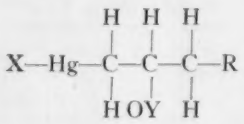
The mercurial diuretics in common use today exhibit certain basic similarities in structure. All are mercurated derivatives of substituted three carbon compounds of the type indicated below, in which the three substituents are designated X, OY, and R.



According to Friedman (1), the nature of the X substituent (usually halogen, theophylline or thioglycolate) has no effect on diuretic potency if the compound is given intravenously, but does influence both hyperacute (cardiac and respiratory arrest) and acute (7 to 14-day) renal toxicity. The nature of the OY group is determined by the solvent in which the mercuration is carried out and is an hydroxyl group, if the solvent is water, or a methoxy or ethoxy group, if the solvent is the corresponding alcohol. Within these limits, *i.e.*, OH, $OCH_3$, or $OC_2H_5$, the nature of the OY group is without appreciable effect on either diuretic potency or toxicity. In contrast, the nature of the R group, which is commonly rather complex, has a very great effect on both toxicity and diuretic activity. In the diuretic mersalyl, R is o-carbamyl-phenoxyacetic acid; in mercaptomerin, R is camphoramic acid; in meralluride, R is succinyl-urea; in chlormerodrin, R is urea; and in mercumatilin, R is coumarin. Much of the interest in the structure-activity relationships of mercurial compounds has centered around the effects of modification of R and relatively less attention has been paid to simple organo-mercurial compounds.

A wide variety of substituted allyl compounds are available or easily prepared as starting materials. Mercuration in methyl alcohol is relatively easy and yields of the 3-mercuri-2-methoxy propyl end products are satisfactory. Logical questions which arise include: Is the three carbon side chain of the presently used diuretics an essential or optimal element of diuretic structure, or is it merely a matter of preparative convenience?

The diuretic activity of organic mercurial compounds has recently been ascribed to inhibition of sulfhydryl enzymes concerned with the renal tubular reabsorption of sodium and chloride ions (2, 3). Mercuric chloride and certain relatively simple organo-mercurials, including bromomercuri-methane, bromo-mercuri-benzene and para-chloromercuri-benzoate, are potent and more or less specific inhibitors of sulfhydryl enzymes *in vitro* (4, 5). Are these compounds also diuretics and are their diuretic activities in any way related to their activities as enzyme inhibitors?

Mercuhydrin® (6, 7) and Neohydrin® (8, 9) have been shown to disappear rapidly from the blood stream following intravenous administration and to accumulate in the renal cortex in high concentration. Both drugs are highly bound to plasma proteins, hence are sparingly filtered through the glomerular capillaries, yet both are rapidly eliminated in the urine. Accordingly, tubular secretion must play a role in their excretion. Is rate of removal from plasma, rapidity of uptake by the renal cortex or rate of secretion by the renal tubules correlated with diuretic activity?

The present study was undertaken to answer the three questions posed above: (a) is the three carbon side chain essential for diuretic action; (b) are sulfhydryl enzyme inhibitors necessarily diuretics; and (c) can diuretic activity be related to distribution and fate of the mercurial compound in the body. Twelve organic compounds of mercury and the single inorganic compound, mercuric chloride, were synthesized in our laboratory using $Hg^{203}$ as a label. Each compound was adminis-

[1] Aided by grants from the National Heart Institute, National Institutes of Health and the Life Insurance Medical Research Fund.

[2] Fellow of the Life Insurance Medical Research Fund.

[3] Fellow of the Rockefeller Foundation.

tered intravenously to anesthetized dogs in doses of 1, 2, and 4 mg. of mercury per kilo of body weight. In all, over 50 experiments have been performed on an equal number of dogs. All experiments were acute for it was necessary to sacrifice each animal in order to obtain tissue samples. If one disregards mercuric chloride, which is moderately effective as a diuretic, the structure which we have observed to be associated with diuretic activity includes (a) a chain of not less than 3 carbon atoms, (b) an atom of mercury attached to the terminal carbon of this chain and (c) some hydrophilic group not less than 3 carbons distant from the mercury. Probably other structures are compatible with diuretic activity; however, many are not. All mercurial compounds are concentrated to some degree in the renal cortex, yet the degree of concentration seems but little related to diuretic potency. Those compounds which are very slowly excreted have no diuretic activity whereas those which are most rapidly excreted are, with one exception, diuretics. However, this exception is sufficiently striking to discourage attaching great significance to a correlation of urinary mercury excretion and diuretic potency. Finally, three of the compounds most widely used as inhibitors of sulfhydryl enzymes *in vitro* are without diuretic activity. Thus, if diuresis is related to inhibition of sulfhydryl enzymes, the inhibitors must have properties other than, or structures different from those of bromomercuri-methane, bromomercuri-benzene and p-chloromercuri-benzoate.

## METHODS

### *Chemical*

All syntheses were performed in our laboratory starting with the radioisotope $Hg^{203}O$. As shipped from Oak Ridge National Laboratories, this product contains 3 curies of $Hg^{197}$ per irradiation unit, with a half life of 2.71 days. To avoid using material of high and rapidly changing activity, the isotopic mixture was allowed to stand for two weeks or more prior to use. The isotope $Hg^{203}$ has a half life of 43.5 days and emits beta particles of 0.205 MEV. and gamma rays of 0.286 MEV. When first used, the organic mercurial compounds counted at a rate of approximately 4,000 per minute per microgram of mercury in a well-type scintillation unit. They were used for two or at most three half life periods; some required recrystallization one or more times during this interval because of decomposition with liberation of metallic mercury, which strangely did not occur with the non-radioactive compounds nor with all of the radioactive ones.

We are indebted to Dr. H. L. Friedman of Lakeside Laboratories, Inc. for directions for the preparation of chlormerodrin and meralluride and also for a supply of both the allylurea and the succinylallylurea used as starting materials. We are similarly indebted to Dr. C. M. Suter, of the Sterling-Winthrop Research Institute for advice on the preparation of mersalyl and for a supply of o-allylcarbamyl-phenoxyacetic acid. The remainder of the syntheses were taken from the literature and the appropriate references are noted in Table I.

All syntheses were performed several times with non-radioactive mercury prior to a radioactive run. Structures of end products were not proved. However, criteria of adequacy of preparation included reproducibility of product, melting point, solubility characteristics and gravimetric mercury analysis as the sulfide. Brief descriptions of these characteristics of the compounds synthesized are included in Table I.

Chemical procedures employed in the analyses of blood, urine and tissue samples are those described in a previous communication (9).

### *Biological*

With one exception,[4] identical procedures were followed in all experiments. All were performed on female mongrel dogs, anesthetized with not more than 30 mg. of pentobarbital per kilo intravenously, supplemented as needed. Following anesthesia, one liter of 0.85 per cent saline was administered intravenously, a priming dose of creatinine was given and an infusion of creatinine in saline was begun at a rate of 2 cc. per minute. The left kidney was exteriorized through a 5-cm. flank incision and held in contact with an end window Geiger tube by a sheet of rubber dam perforated to pass the renal pedicle. A film of Saran plastic separated the kidney from the Geiger tube to protect the latter from contamination. Counts were recorded every minute and represent semi-quantitatively variations in the concentration of radiomercury in the outer few millimeters of the renal cortex. When urine flow stabilized, two 20-minute control clearance periods were obtained. Blood samples were drawn through a retention needle in the femoral artery, urine was collected with a Foley catheter and the bladder was washed out with 10 cc. of distilled water at the end of each urine collection period.

The mercury compound, dissolved with a minimum of alkali or alcohol, if the solubility was greater in the latter solvent, was administered intravenously in 20 seconds following the second control clearance period. Each compound was given in three experiments in doses of 1, 2 and 4 mg. mercury per kilo body weight. Arterial blood samples were drawn 1, 2, 3, 5, 7, 10 and 15 minutes after the start of the mercury injection and thereafter

[4] In this experiment, designed to test the effects of Probenecid® on the secretion of p-chloromercuri-benzoate, the mercurial compound was infused intravenously throughout the course of the experiment instead of being administered rapidly in a single intravenous injection.

at the mid-point of each urine collection period. The first two clearance periods following mercury were 10 minutes in length. Thereafter, 20-minute collections were continued for a total of 3 hours.

At the end of the experiment, the animal was sacrificed and samples of heart, lung, diaphragm, gut, skin, liver and spleen were removed. One kidney was cored from cortex through medullary papilla with a No. 5 cork borer. The cylinder of tissue so obtained was sliced free hand with a razor blade into nine or ten sections as nearly equal in thickness as possible. Usually three sections were obtained from the cortex, three from the medulla and four from the papilla. The kidney sections (weighing 15 to 60 mg.) and samples of the other tissues of appropriate size were placed in tared test tubes, weighed wet and counted without further preparation in a well-type scintillation unit. Plasma binding of each mercurial compound was estimated by high pressure ultrafiltration of plasma through cellophane.

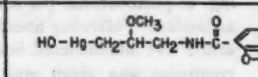
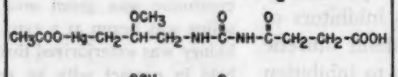
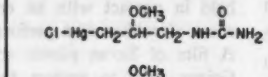
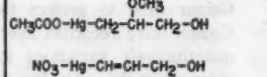
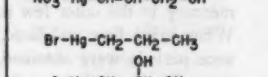
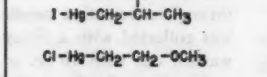
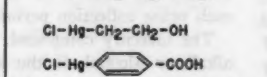
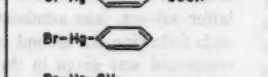
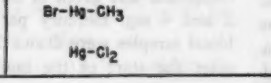
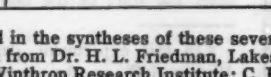
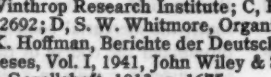
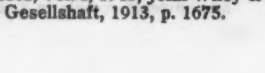
## RESULTS

The 13 compounds studied are listed in Table I in order of decreasing complexity, modified to place last the four compounds used *in vitro* as sulfhydryl enzyme inhibitors. When arranged in this order, the first five compounds have diuretic properties, the next seven are not diuretics, and the final one, mercuric chloride, is again a diuretic.

We have taken as an indication of diuretic activity, an increase in urine flow or an increase in sodium excretion with respect to the two control periods in any period within three hours after the intravenous injection of an amount of the compound containing 1, 2, or 4 mg. of mercury. We have been fortunate in the application of these cri-

**TABLE I**

***Structures, physical properties and methods of preparation and purification of the one inorganic and twelve organic compounds of mercury used in these studies ****

| COMPOUND | FORMULA | RECRYSTALLIZATION SOLVENT | MELTING POINT | | % MERCURY | | REFERENCE |
|---|---|---|---|---|---|---|---|
| | | | Reported | Found | Calc. | Obs. | |
| 3-Hydroxymercuri-2-methoxy-1-propylcarbamyl-O-phenoxy-acetate (Mersalyl) |  | Water | — | — | 41.6 | 42.6 | A |
| 3-Acetomercuri-2-methoxy-succinyl-propylurea (Meralluride) |  | Ethanol | 177-8½ | 178 | 40.9 | 42.8 | B |
| 3-Chloromercuri-2-methoxy-propylurea (Chlormerodrin) |  | Ethanol | 152-3 | 153 | 54.6 | 54.7 | B |
| 3-Acetomercuri-2-methoxy-1-hydroxy-propane |  | Methanol | — | 162 | 57.6 | 58.2 | B |
| 3-Nitromercuri-2,3-dehydro-1-hydroxy-propane |  | Water | 271 | 260 | 62.8 | 62.8 | C |
| 3-Bromomercuri-propane |  | Ethanol-Water | 134 | 132-5 | — | — | D |
| 3-Iodomercuri-2-hydroxy-propane |  | Ethanol-Water | 68 | 68 | 55.4 | 56.0 | E |
| 2-Chloromercuri-1-methoxy-ethane |  | Water | 68 | 68 | 68.1 | 70.0 | D |
| 2-Chloromercuri-1-hydroxy-ethane |  | Methanol | 155-6 | 157 | 73.0 | 73.3 | D |
| p-Chloromercuri-benzoate |  | Water | 273 | 273 | — | — | F |
| Bromomercuri-benzene |  | Pyridine | 275 | 274-6 | — | — | G |
| Bromomercuri-methane |  | Ethanol-Water | 160 | 165 | — | — | D |
| Mercuric Chloride |  | Water | — | — | — | — | — |

* Procedures employed in the syntheses of these several compounds were derived from the following sources: A, Personal communication from Dr. H. L. Friedman, Lakeside Laboratories, Inc.; B, personal communication from Dr. C. M. Suter, Sterling-Winthrop Research Institute; C, K. Hoffman and J. Sand, Berichte der Deutschen Chemischen Gesellshaft, 1900, p. 2692; D, S. W. Whitmore, Organic Compounds of Mercury, 1921, Chemical Catalogue Co., New York; E, J. Sand and K. Hoffman, Berichte der Deutschen Chemischen Gesellshaft, 1900, p. 1353; F, H. Gilman and A. Blatt, Organic Syntheses, Vol. I, 1941, John Wiley & Sons, New York; G, S. Hilpert and G. Gruttner, Berichte der Deutschen Chemischen Gesellshaft, 1913, p. 1675.

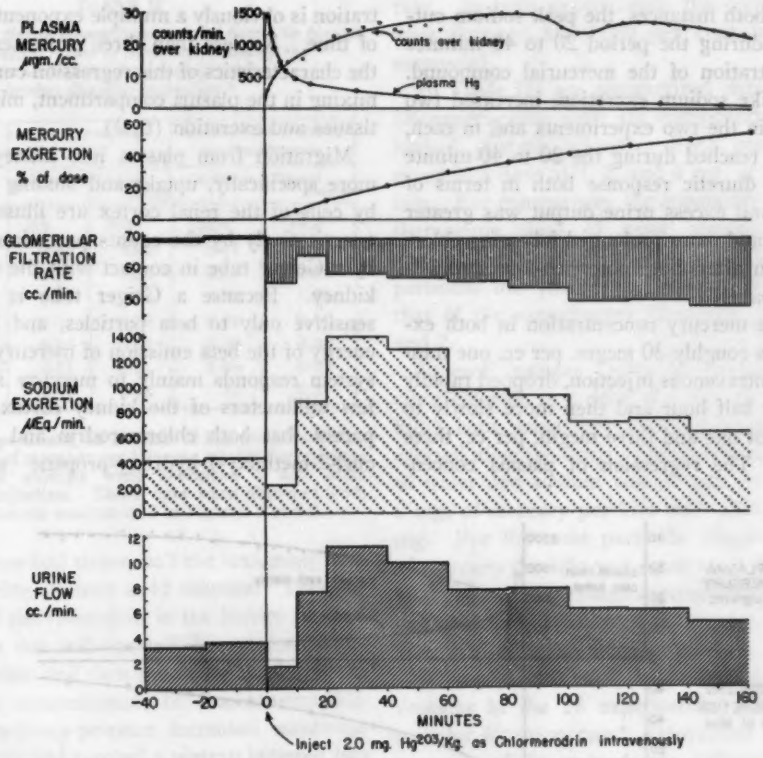



FIG. 1. SUMMARY OF DATA OBTAINED IN AN EXPERIMENT IN WHICH A DIURETIC COMPOUND, CHLORMERODRIN, WAS ADMINISTERED INTRAVENOUSLY IN A DOSE OF 2.0 MG. OF MERCURY PER KILO

See text for further description.

teria to find that any compound increasing urine flow also increased sodium excretion; that any compound (except $HgCl_2$)[5] which has a diuretic action in one dose, exhibited such an action in all three doses; and that any compound which significantly increased urine flow or sodium excretion in one 20-minute collection period, did so in several consecutive periods. There has, therefore, been no difficulty in deciding, according to these criteria, whether a compound is or is not a diuretic. These criteria are, of course, remarkably restrictive, especially with respect to time, but we feel, by defining diuresis in such terms in the dog, we are studying a phenomenon related to that observed in the successful therapy of edematous patients. We are not convinced that the "diuresis" observed in rabbits by Fourneau and Melville (10), having its onset three days after a single intravenous injection, has any bearing on diuretic therapy.

Figures 1 and 2 summarize experiments in which two organo-mercurial compounds having diuretic properties were administered in doses of 2 mg. of mercury per kilo of body weight: Figure 1, chlormerodrin; Figure 2, 3-acetomercuri-2-methoxy-1-hydroxy-propane.

The significant features of the diuretic responses in these experiments are the following: sodium excretion increased from a mean of 427 mEq. per minute before, to a peak of 1,395 mEq. per minute after chlormerodrin and from a mean of 423 mEq. per minute before, to a peak of 1,040 mEq. per minute after 3-acetomercuri-2-methoxy-1-hydroxy-

[5] The single exception refers to the fact that mercuric chloride in the highest dose (4 mg. of mercury per kilo) caused a drop in urine flow and in sodium excretion in association with a collapse of filtration rate.

propane. In both instances, the peak sodium output occurred during the period 20 to 40 minutes after administration of the mercurial compound. Urine flow, like sodium excretion, increased two to three fold in the two experiments and in each, the peak was reached during the 20 to 40-minute period. The diuretic response both in terms of sodium loss and excess urine output was greater in magnitude and more prolonged following chlormerodrin than after 3-acetomercuri-2-methoxy-1-hydroxy-propane.

The plasma mercury concentration in both experiments was roughly 30 mcgm. per cc. one minute after the intravenous injection, dropped rapidly over the first half hour and then more slowly to reach values of one and three mcgm. per cc. three hours later. The regression of plasma concentration is obviously a multiple exponential function of time. No less than three processes determine the characteristics of this regression curve, namely, mixing in the plasma compartment, migration into tissues and excretion (6, 9).

Migration from plasma into kidney tissue or, more specifically, uptake and binding of mercury by cells of the renal cortex are illustrated semiquantitatively by the counts per minute recorded by a Geiger tube in contact with the exteriorized kidney. Because a Geiger tube is appreciably sensitive only to beta particles, and because the energy of the beta emission of mercury is low, the system responds mainly to mercury in the outer few millimeters of the kidney cortex. It is apparent that both chlormerodrin and 3-acetomercuri-2-methoxy-1-hydroxy-propane are rapidly

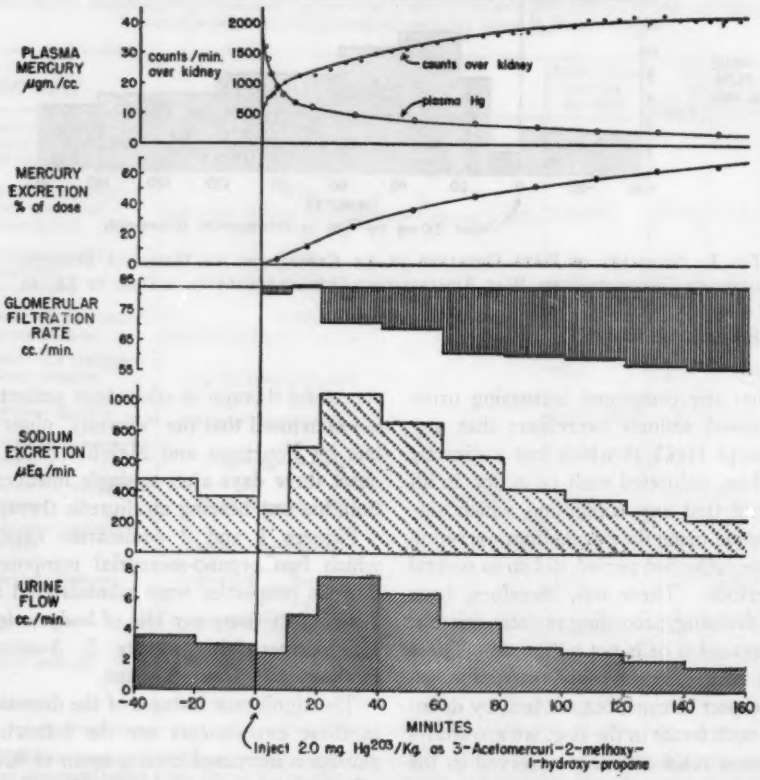



FIG. 2. SUMMARY OF DATA OBTAINED IN AN EXPERIMENT IN WHICH A DIURETIC COMPOUND, 3-ACETOMERCURI-2-METHOXY-1-HYDROXY-PROPANE, WAS ADMINISTERED INTRAVENOUSLY IN A DOSE OF 2.0 MG. OF MERCURY PER KILO

See text for further description.

TABLE II

*Comparison of tissue concentrations and tissue to plasma concentration ratios of mercury following the administration of two compounds having diuretic properties* *

| | Chlormerodrin | | 3-Acetomercuri-2-Methoxy-1-Hydroxy-Propane | |
|---|---|---|---|---|
| | μgm. Hg/gm. | Tissue/Plasma | μgm. Hg/gm. | Tissue/Plasma |
| Kidney Cortex | 155 | 144 | 38.9 | 12.2 |
| Liver | 3.89 | 3.60 | 5.45 | 1.70 |
| Spleen | 1.39 | 1.29 | 3.16 | 0.99 |
| Lung | 1.23 | 1.20 | 4.32 | 1.35 |
| Heart | 0.29 | 0.28 | 2.59 | 0.80 |
| Gut | 0.70 | 0.66 | 2.59 | 0.80 |
| Diaphragm | 0.23 | 0.21 | 0.84 | 0.26 |
| Skin | 0.40 | 0.35 | 1.95 | 0.61 |

* In each experiment, an amount of the compound containing 2.0 mg. of mercury per kilo was administered intravenously. The animals were sacrificed 3 hrs. after receiving the injection. These data were obtained from the two experiments summarized in Figures 1 and 2.

taken up by cortical tissue, half the maximum concentration being attained in 15 minutes. The concentration of chlormerodrin in the kidney reached a peak about one and one-half hours after intravenous injection and then gradually declined. In contrast, the concentration of 3-acetomercuri-2-methoxy-1-hydroxy-propane increased somewhat more gradually and reached a plateau between two and three hours after injection.

The concentration of mercury in the renal cortex and in representative tissues at the end of each of these two experiments are given in Table II. It is evident that the kidney concentrates each substance highly with respect to plasma, that the liver, spleen and lung concentrate them very slightly and that the remaining tissues do not concentrate them at all.

The rate of excretion of mercury, in per cent of dose administered, and the glomerular filtration rate, as measured by the creatinine clearance, are also given in Figures 1 and 2. Excretion of mercury was rapid following both mercurial compounds and in the two hours and forty minutes following intravenous administration; 54.9 per cent of the chlormerodrin and 66.6 per cent of the 3-acetomercuri-2-methoxy-1-hydroxy-propane were eliminated in the urine. The two compounds exhibited equivalent toxicity in a dose of 2 mg. of mercury per kilo when toxicity is expressed in terms of depression of glomerular filtration rate. In both experiments, the creatinine clearance dropped about 30 per cent following the mercurial.

In Table III are summarized data obtained in 18 experiments with six compounds which exhibited diuretic activity. Each compound was given in a dose of 1, 2, and 4 mg. of mercury per kilo. For comparison, the commonly used human therapeutic dose of most mercurial diuretics amounts to 1 mg. of mercury per kilo. Each experiment was performed in a manner similar to that of the experiments illustrated in Figures 1 and 2.

In each instance (with the exception of mercuric chloride in a dose of 4 mg. of mercury per kilo) both urine flow and sodium excretion increased after the drug. Furthermore, the diuresis of water and sodium was greater after a dose of 2 mg. of mercury per kilo than after a dose of 1 mg. For the most part, the response to 4 mg. of mercury per kilo was equal to or less than that to 2 mg. Only 3-acetomercuri-2-methoxy-1-hydroxy-propane exhibited a greater diuretic response to 4 mg. of mercury per kilo.

All compounds in all dose ranges (with two exceptions in the 18 experiments) depressed glomerular filtration rate.[6] Depression for the most part was slight to moderate, although greater in the higher dosage range. However, what must have been individual differences in susceptibility complicated the picture. Of all the compounds studied, 3-acetomercuri-2-methoxy-succinylpropylurea appeared most benign, mercuric chloride most toxic.

All of the diuretic compounds of mercury shown in Table III are more or less rapidly eliminated in the urine. From 10 to 70 per cent of the dose was excreted in the two hours and forty minutes after intravenous administration. The clearance[7] of mercury ranged from 7 to 45 cc. per minute. Since these compounds were from 99.4 to essentially 100 per cent bound by plasma proteins, they

[6] Depression of filtration rate is no doubt due in part to the rapidity of intravenous injection of the diuretic, *i.e.*, 20 seconds.

[7] Because of the rapid uptake of the mercurial compounds by the renal tubules early in the course of the experiment, their combination with cysteine in the tubular cells and their secretion into the urine, clearance has a somewhat different connotation than it usually has.

TABLE III

*Summary of results obtained in 18 experiments in which six diuretic compounds were administered intravenously in doses of 1, 2 and 4 mg. of mercury per kilo* *

| | Dose | Urine Flow | | Na Excretion | | Creatinine Clearance | | Mercury % Dose Excr. | Mercury $C_{Hg}$ cc./min. | Terminal Mercury Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg. Hg /Kg. | Cont. cc./min. | Max. cc./min. | Cont. μEq./min. | Max. μEq./min. | Cont. cc./min. | Max. cc./min. | | | Plasma μgm./cc. | Renal Cortex μgm./gm. | Spleen μgm./gm. | Liver μgm./gm. | Diaph. μgm./gm. |
| 3-Hydroxymercuri-2-methoxy-1-propylcarbamyl-o-phenoxy-acetate (Mersalyl) | 1 | 1.1 | 1.9 | 190 | 400 | 62 | 67 | 67 | 34 | 0.5 | 9 | 0.1 | 0.6 | 0.2 |
| | 2 | 2.7 | 8.5 | 360 | 980 | 64 | 52 | 62 | 25 | 1.2 | 10 | 1.1 | 0.8 | 0.1 |
| | 4 | 3.2 | 4.8 | 130 | 540 | 43 | 28 | 46 | 20 | 2.4 | 19 | 1.8 | 2.5 | 0.4 |
| 3-Acetomercuri-2-methoxy-succinyl-propylurea (Meralluride) | 1 | 1.8 | 4.8 | 270 | 630 | 68 | 62 | 59 | 26 | 1.0 | 9 | 0.6 | 0.7 | 0.2 |
| | 2 | 3.1 | 7.9 | 230 | 910 | 64 | 53 | 74 | 34 | 1.3 | 22 | 0.6 | 0.8 | 0.8 |
| | 4 | 3.2 | 7.3 | 560 | 1160 | 66 | 71 | 61 | 20 | 6.0 | 72 | 2.4 | 3.7 | 0.7 |
| 3-Chloromercuri-2-methoxy-propylurea (Chlormerodrin) | 1 | 1.6 | 10. | 150 | 1290 | 51 | 45 | 48 | 26 | 0.8 | 75 | 0.3 | 2.2 | 0.2 |
| | 2 | 3.5 | 11. | 420 | 1340 | 69 | 49 | 55 | 45 | 1.1 | 150 | 1.4 | 3.4 | 0.2 |
| | 4 | 3.5 | 6.5 | 98 | 760 | 71 | 34 | 26 | 22 | 5.2 | 390 | 9.1 | 8.5 | 0.8 |
| 3-Acetomercuri-2-methoxy-1-hydroxy-propane | 1 | 4.4 | 6.8 | 720 | 930 | 84 | 72 | 53 | 15 | 1.2 | 15 | 2.4 | 2.3 | 0.3 |
| | 2 | 3.3 | 6.9 | 430 | 950 | 83 | 57 | 67 | 15 | 3.2 | 39 | 3.2 | 5.5 | 0.8 |
| | 4 | 3.6 | 8.8 | 370 | 1020 | 68 | 43 | 41 | 12 | 5.7 | 45 | 13. | 9.4 | 1.3 |
| 3-Nitromercuri-3-2-dehydro-1-hydroxy-propane | 1 | 1.3 | 4.1 | 140 | 520 | 43 | 28 | 52 | 7.2 | 2.1 | 31 | 3.5 | 3.8 | 0.6 |
| | 2 | 3.0 | 9.6 | 420 | 1300 | 65 | 50 | 10 | 7.4 | 2.1 | 23 | 24. | 4.8 | 0.6 |
| | 4 | 2.0 | 8.8 | 200 | 1190 | 53 | 45 | 35 | 9.6 | 10. | 68 | 60. | 17. | 1.7 |
| Mercuric Chloride | 1 | 4.2 | 6.9 | 470 | 850 | 58 | 48 | 23 | 9.0 | 1.6 | 54 | 40. | 5.1 | 0.2 |
| | 2 | 3.2 | 8.4 | 450 | 1260 | 53 | 35 | 21 | 9.0 | 2.9 | 120 | 74. | 6.7 | 0.3 |
| | 4 | 0.8 | 0.4 | 92 | 37 | 45 | 2 | 4 | 1.8 | 8.3 | 240 | 31. | 13. | 1.2 |

* Urine flow, sodium excretion and creatinine clearance are presented as averages of the values observed in the two control periods and in the two successive experimental periods in which maximum changes occurred. Mercury excretion is presented both in terms of per cent of the administered dose eliminated in the 3 hrs. following intravenous injection of the diuretic and in terms of the maximum plasma clearance of mercury. In the last 5 columns on the right are shown the mercury concentrations of plasma and of four representative tissues observed at the end of the experiment.

We have no explanation for the aberrant result on mercury excretion obtained in the experiment in which 2.0 mg. of mercury was administered as 3-nitromercuri-3-2-dehydro-1-hydroxy-propane. Only 10 per cent of the administered dose was excreted in comparison with the 52 and 35 per cent excreted at lower and higher dose levels. The clearance, however, is correctly tabulated for plasma concentration was proportionately as low as urinary excretion.

must have been in large part actively secreted into the tubular urine, for no significant amount is free to enter the glomerular filtrate.

The concentration of mercury in the plasma and in four representative tissues at the end of each of the several experiments are shown on the right of Table III. All of the diuretic compounds are more highly concentrated in the renal cortex than in any other tissue, the greatest absolute concentration of mercury being attained following a dose of 3-chloromercuri-2-methoxypropyl-urea (chlormerodrin) equivalent to 4 mg. of mercury per kilo. The concentration attained in this experiment was 390 mcgm. per gm., roughly $2 \times 10^{-3}$ molar mercury. Significant concentrations of mercury were attained in the spleen and liver. Two compounds, namely 3-nitromercuri-2-3-dehydro-1-hydroxy-propane and mercuric chloride were rather highly concentrated in the spleen. Lung, heart, gut, skin and diaphragm concentrated the mercury slightly or not at all. Least active in this respect was diaphragm, shown in the last column of Table III.

Figures 3 and 4 summarize experiments in which two organo-mercurial compounds exhibiting no diuretic activity were administered in doses of

2 mg. of mercury per kilo of body weight: Figure 3, p-chloromercuri-benzoate; Figure 4, 3-iodomercuri-2-hydroxy-propane. Urine flow and sodium excretion decreased after the injection of both compounds, similar decreases occurring at all dose levels within the range of 1 and 4 mg. of mercury per kilo. Although this antidiuresis in the p-chloromercuri-benzoate experiment was associated with a modest fall in filtration rate, no such fall was observed following 3-iodomercuri-2-hydroxy-propane. Furthermore, following doses of 1 and 4 mg. per kilo of mercury as p-chloromercuri-benzoate, lesser falls in filtration rate occurred with equivalent antidiureses.

The plasma concentration of mercury decreased from a peak of 31 mcgm. per cc. one minute after the intravenous injection of p-chloromercuri-benzoate to a low of 4 mcgm. per cc. two hours and fifty minutes later. The plasma regression curve was very similar to that following 3-acetomercuri-2-methoxy-1-hydroxy-propane. In contrast the regression curve following 3-iodomercuri-2-hydroxy-propane was very different, the peak value one minute after injection amounting only to 2.78 mcgm. per cc. A plateau of 0.4 mcgm. per cc. was attained after an hour and was maintained for the remainder of the experiment.

Uptake of mercury by the renal cortex, as measured by a Geiger tube in contact with the kidney surface, was very rapid in both experiments; half the peak concentration of each compound was attained within 1 to 2 minutes. However, the concentration of p-chloromercuri-benzoate reached a plateau early, whereas that of 3-iodomercuri-2-hydroxy-propane continued to rise throughout the experiment. Early plateau-

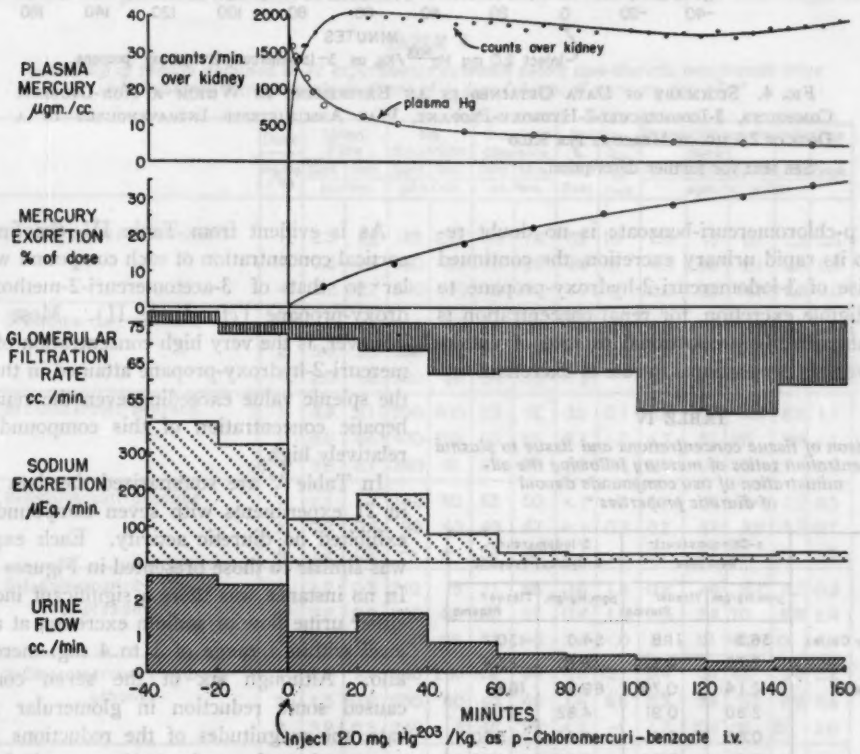



FIG. 3. SUMMARY OF DATA OBTAINED IN AN EXPERIMENT IN WHICH A NON-DIURETIC COMPOUND, P-CHLOROMERCURI-BENZOATE, WAS ADMINISTERED INTRAVENOUSLY IN A DOSE OF 2.0 MG. OF MERCURY PER KILO

See text for further description.

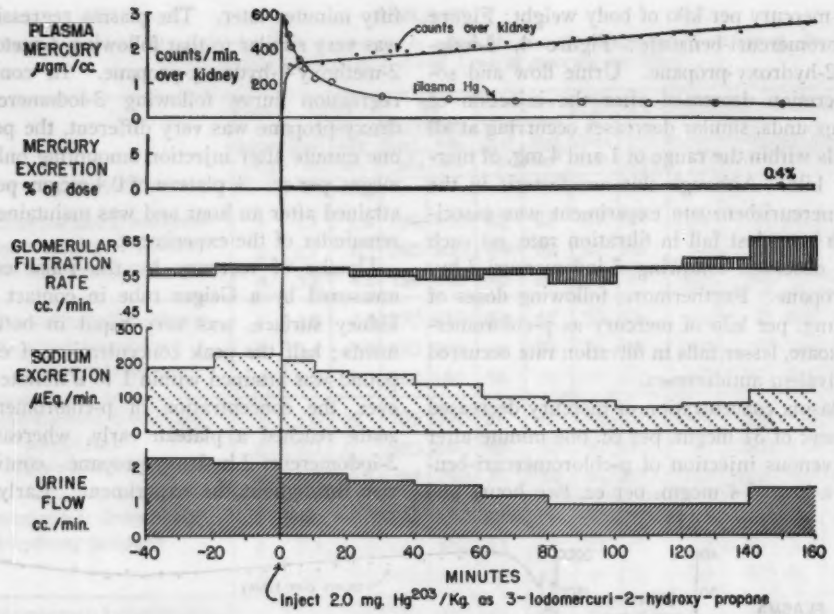



FIG. 4. SUMMARY OF DATA OBTAINED IN AN EXPERIMENT IN WHICH A NON-DIURETIC COMPOUND, 3-IODOMERCURI-2-HYDROXY-PROPANE, WAS ADMINISTERED INTRAVENOUSLY IN A DOSE OF 2.0 MG. OF MERCURY PER KILO

See text for further description.

ing of p-chloromercuri-benzoate is no doubt related to its rapid urinary excretion, the continued slow rise of 3-iodomercuri-2-hydroxy-propane to its negligible excretion, for renal concentration is no doubt directly proportional to rate of uptake and inversely proportional to rate of excretion.

TABLE IV

*Comparison of tissue concentrations and tissue to plasma concentration ratios of mercury following the administration of two compounds devoid of diuretic properties* *

| | p-Chloromercuri-Benzoate | | 3-Iodomercuri-2-Hydroxy-Propane | |
|---|---|---|---|---|
| | µgm. Hg/gm. | Tissue/Plasma | µgm. Hg/gm. | Tissue/Plasma |
| Kidney Cortex | 36.3 | 11.8 | 54.0 | 130 |
| Liver | 2.79 | 0.91 | 9.75 | 23.4 |
| Spleen | 2.14 | 0.70 | 69.6 | 168 |
| Lung | 2.80 | 0.91 | 4.82 | 11.6 |
| Heart | 0.66 | 0.21 | 1.45 | 3.50 |
| Gut | 2.47 | 0.80 | 2.00 | 4.80 |
| Diaphragm | 0.76 | 0.25 | 2.38 | 5.72 |
| Skin | 0.96 | 0.31 | 1.05 | 2.52 |

* See legend of Table II for further clarification.

As is evident from Table IV, the final renal cortical concentration of each compound was similar to that of 3-acetomercuri-2-methoxy-1-hydroxy-propane (*cf.*, Table II). Most striking, however, is the very high concentration of 3-iodomercuri-2-hydroxy-propane attained in the spleen, the splenic value exceeding even the renal. The hepatic concentration of this compound is also relatively high.

In Table V are summarized the data obtained in 21 experiments with seven compounds which exhibited no diuretic activity. Each experiment was similar to those presented in Figures 3 and 4. In no instance was there a significant increase in either urine flow or sodium excretion at any dose level within a range of 1 to 4 mg. mercury per kilo. Although six of the seven compounds caused some reduction in glomerular filtration rate, the magnitudes of the reductions were no greater than those observed to result from diuretic compounds (*cf.*, Table III). Furthermore, each non-diuretic compound in at least one dose level failed to alter filtration rate and 2-chloro-

mercuri-1-methoxy-ethane was remarkably benign at all dose levels.

If one excludes p-chloromercuri-benzoate, all non-diuretic compounds studied were poorly excreted whereas all diuretic compounds were rapidly eliminated in the urine. Because high rate of excretion seemed so clearly related to diuretic action with the single exception of p-chloromercuri-benzoate, it was felt that this latter compound might be secreted by a different mechanism. The inference was that secretion of the diuretic compounds concentrates them at a mercury sensitive site of salt reabsorption. P-chloromercuri-benzoate, secreted by another mechanism and concentrated at another site, might thus be devoid of diuretic properties. We felt it at least possible that p-chloromercuri-benzoate might be secreted by the mechanism which transports Diodrast®, para-aminohippurate and penicillin and thus be sensitive to Benemid®. Evidence obtained in two experiments clearly demonstrated that Probenecid had no effect on renal uptake, regression of plasma concentration or rate of urinary excretion of p-chloromercuri-benzoate. We therefore have no evidence that this latter compound is secreted by a mechanism different from that of the diuretic compounds. Its high rate of excretion remains an exception to any generalization relating this property to diuretic activity.

It is evident from Tables III and V that all compounds, both diuretic and non-diuretic, are concentrated in the renal cortex. Although chlormerodrin, which is the most active of the diuretics studied, is also most highly concentrated in the kidney, the degree of concentration of the remaining compounds seems entirely unrelated to diuretic potency. Figure 5 illustrates the distribution of mercury in the kidney in four experiments in

TABLE V

*Summary of results obtained in 21 experiments in which seven non-diuretic compounds were administered intravenously in doses of 1, 2 and 4 mg. of mercury per kilo* *

| | Dose | Urine Flow | | Na Excretion | | Creatinine Clearance | | Mercury | | Terminal Mercury Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | % | $C_{Hg}$ | | Renal | | | |
| | mg. Hg / Kg. | Cont. | Max. | Cont. | Max. | Cont. | Max. | Dose Excr. | cc. /min. | Plasma | Cortex | Spleen | Liver | Diaph. |
| | | cc./min. | | μEq./min. | | cc./min. | | | | μgm./cc. or /gm. | | | | |
| p-Chloromercuri-benzoate | 1 | 2.3 | 1.2 | 185 | 210 | 89 | 76 | 38 | 10 | 2.3 | 13 | 0.7 | 1.3 | 0.3 |
| | 2 | 2.5 | 0.3 | 350 | 20 | 76 | 51 | 36 | 12 | 4.1 | 36 | 2.1 | 2.8 | 0.8 |
| | 4 | 3.2 | 2.7 | 540 | 140 | 48 | 42 | 44 | 9 | 10.0 | 95 | 4.7 | 7.9 | 1.0 |
| Bromomercuri-methane | 1 | 1.3 | 0.5 | 140 | 20 | 73 | 50 | 0.2 | 0.2 | 0.3 | 5 | 7.9 | 2.3 | 0.6 |
| | 2 | 5.0 | 3.6 | 380 | 360 | 55 | 50 | <.1 | 0.2 | 0.6 | 21 | 30. | 9.2 | 1.5 |
| | 4 | 2.5 | 1.2 | 380 | 66 | 34 | 25 | <.1 | — | 0.5 | 27 | 40. | 19. | 2.4 |
| Bromomercuri-benzene | 1 | 4.8 | 3.1 | 590 | 500 | 53 | 46 | 0.2 | 0.1 | 0.8 | 36 | 7.3 | 6.2 | 1.1 |
| | 2 | 5.6 | 4.6 | 470 | 250 | 63 | 55 | 0.7 | 1.0 | 1.7 | 51 | 20. | 9.8 | 1.1 |
| | 4 | 5.5 | 2.1 | 360 | 51 | 67 | 35 | 2.7 | 4.5 | 2.5 | 97 | 18. | 27. | 2.2 |
| Bromomercuri-propane | 1 | 1.1 | 1.7 | 140 | 60 | 63 | 50 | <.1 | — | 0.1 | 18 | 5.6 | 3.7 | 0.5 |
| | 2 | 0.8 | 0.4 | 96 | 40 | 49 | 47 | <.1 | 0.3 | 0.2 | 37 | 8.2 | 5.7 | 0.7 |
| | 4 | 1.2 | 0.2 | — | — | 58 | 30 | <.1 | — | 0.7 | 27 | 23. | 15. | 1.3 |
| 3-Iodomercuri-2-hydroxy-propane | 1 | 1.2 | 0.3 | 210 | 15 | 37 | 25 | 0.6 | 1.3 | 0.2 | 24 | 2.7 | 2.7 | 0.3 |
| | 2 | 2.2 | 0.9 | 200 | 80 | 57 | 57 | 0.4 | 1.5 | 0.4 | 54 | 70. | 9.8 | 2.4 |
| | 4 | 2.1 | 1.8 | 240 | 96 | 43 | 40 | 0.4 | 1.0 | 1.1 | 91 | 160. | 15. | 1.8 |
| 2-Chloromercuri-1-hydroxy-ethane | 1 | 2.6 | 1.1 | 560 | 250 | 102 | 90 | 6.6 | 13. | 0.4 | 56 | 13. | 3.0 | 0.2 |
| | 2 | 1.5 | 0.6 | 230 | 60 | 45 | 46 | 1.4 | 6.5 | 0.3 | 39 | 8.9 | 3.6 | 0.3 |
| | 4 | 2.9 | 0.3 | 260 | 3 | 48 | 25 | <.1 | — | 1.7 | 104 | 35. | 22. | 2.0 |
| 2-Chloromercuri-1-methoxy-ethane | 1 | 1.9 | 0.8 | 450 | 96 | 77 | 72 | 2.9 | 9.0 | 0.2 | 35 | 16. | 2.1 | 0.8 |
| | 2 | 1.5 | 0.7 | 150 | 54 | 77 | 73 | 0.5 | 1.7 | 0.4 | 59 | 38. | 5.9 | 1.0 |
| | 4 | 1.7 | 1.1 | 93 | 124 | 70 | 70 | 0.2 | 1.0 | 0.6 | 152 | 62. | 19. | 2.3 |

* See legend of Table III for further clarification.

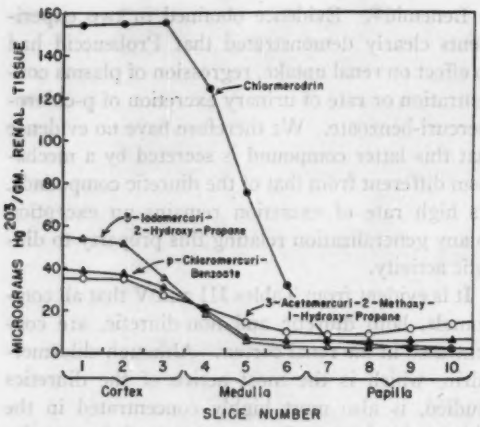



FIG. 5. A COMPARISON OF THE DISTRIBUTIONS OF MERCURY IN THE CORTEX, MEDULLA AND PAPILLA OF THE KIDNEY FOLLOWING THE INTRAVENOUS ADMINISTRATION OF TWO DIURETIC COMPOUNDS, CHLORMERODRIN AND 3-ACETOMERCURI-2-METHOXY-1-HYDROXY-PROPANE, AND TWO NON-DIURETIC COMPOUNDS, P-CHLOROMERCURI-BENZOATE AND 3-IODOMERCURI-2-HYDROXY-PROPANE

In each experiment an amount of the compound containing 2.0 mg. of mercury per kilo was administered 3 hrs. before sacrificing the animal to obtain the renal tissue. See text for description of method of sampling. Data derived from experiments summarized in Figures 1, 2, 3 and 4.

which 2 mg. of mercury per kilo were administered as chlormerodrin, 3-acetomercuri-2-methoxy-1-hydroxy-propane, p-chloromercuri-benzoate and 3-iodomercuri-2-hydroxy-propane. All compounds exhibited a qualitatively similar distribution, being most highly concentrated in the cortex, less in the medulla and least in the papilla. The last three of these compounds exhibited a quantitatively similar distribution, the two non-diuretics bracketing closely the diuretic. Thus, neither total concentration of the compound nor peculiarity of distribution could be related to diuretic activity.

## DISCUSSION

A number of studies have demonstrated that mercurial diuretics are accumulated in kidney tissue and that diuresis occurs only after some undefined threshold concentration has been achieved (9). Results presented in this paper demonstrate that non-diuretic compounds of mercury are likewise concentrated in kidney tissue and that their distribution within renal cortex, medulla and papilla is both qualitatively and quantitatively similar to that of diuretic compounds. Accordingly, binding by renal tissue is not a feature which distinguishes diuretic from non-diuretic compounds of mercury.

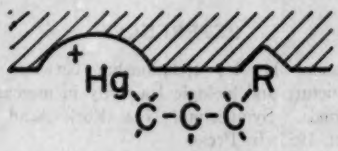
It is generally believed that the combination of inorganic mercury, split off from the diuretic, or of the free mercury bond of the intact diuretic molecule with renal sulfhydryl enzymes renders those enzymes inactive and results in diuresis (2). Recent evidence supports the thesis that the intact molecule rather than inorganic mercury is the active agent (11, 12). Our results demonstrate that compounds active *in vitro* as inhibitors of sulfhydryl enzymes are not necessarily diuretics *in vivo*. Thus of the four commonly used inhibitors of sulfhydryl enzymes *in vitro*, three, namely bromomercuri-methane, bromomercuri-benzene and p-chloromercuri-benzoate, have no diuretic activity *in vivo*. If inhibition of sulfhydryl enzymes is a significant element of the diuretic process, then the inhibitor must have properties other than, or in addition to, those of the three compounds mentioned above.

All of the mercurial compounds which we have studied have been so highly bound to the plasma proteins that little could be filtered through the glomeruli; most of that which enters the urine must have been eliminated by tubular secretion. Of the 13 compounds studied, 6 with diuretic properties are rapidly excreted, while 6 of the 7 without diuretic properties are slowly excreted. One might infer that active tubular secretion of a mercurial compound leads to its accumulation in a region where enzymes significant for the reabsorption of salt and water are located. Blockade of these enzymes results in diuresis. Against this view is our finding that p-chloromercuri-benzoate, which is not a diuretic, is rapidly excreted in the urine, presumably by the same mechanism responsible for the secretion of the several diuretic agents.

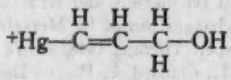
More or less by exclusion, we have been forced to the view that steric configuration is significant in determining whether or not a given compound of mercury will have diuretic properties. We suggest that some "critical lock and key" relationship [8]

[8] Steric configuration might be related to diuretic activity in a number of ways other than by the mechanism postulated. Thus a hydrophilic group three carbons dis-

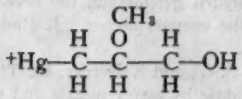
exists which can be schematically represented as follows:




The simplest compounds which we have observed as having diuretic properties include the following:

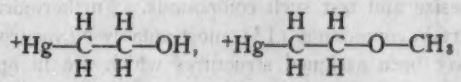



and

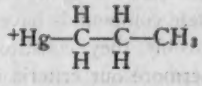



These compounds exhibit a terminal mercury-carbon linkage, a free mercury valence and a hydrophilic substituent (in these instances an OH group) three carbons removed from the mercury. As pointed out in the introduction, this configuration characterizes the mercurial diuretics in common use today although the hydrophilic substituent is more complex than that of the compounds noted above. The simplest hydrophilic substituent in a clinically useful diuretic is urea, present in chlormerodrin.

The non-diuretic compounds most closely related to the two diuretics described above have the following structures:

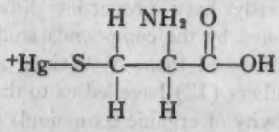



and




The first two differ in having a two carbon rather than a three carbon chain. The third differs in having no hydrophilic substituent on the terminal carbon. Obviously to determine whether the three carbon chain is truly significant, it would be necessary to show that a 4-mercurated-n-butanol is not diuretic. To date we have been unable to synthesize this compound.

If steric configuration is significant in determining whether or not an organic compound of mercury will exhibit diuretic activity, why is bichloride of mercury a moderately effective diuretic? A simple answer might be that inorganic mercury binds readily to a single active enzyme site. Only when an organic side chain is attached to one mercury valence, does it become necessary to have a second polar binding group three carbons distant from the mercury in order to form a stable complex with the enzyme. Another possibility is that the diuretic substance is not mercuric ion but a monothiol complex of mercury. Weiner and Müller (11) have shown that Salyrgan® and chlormerodrin are excreted in combination with cysteine and acetylcysteine. Were mercuric ion to complex with cysteine in a 1:1 molar ratio in the kidney, the resulting compound would exhibit in some respects the features of a diuretic as outlined above:




If it be permissible to replace carbon with sulfur, the chain of one sulfur and two carbons between the mercury and the hydrophilic amino group would constitute the usual three units.

In all fairness, we must point out that this hypothesis, which imputes diuretic significance to a three unit chain linking mercury and a hydrophilic group, is a tenuous one. It is possible that a chain of four or more units would be compatible with

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tant might increase the dissociation of the terminal atom of mercury as free mercuric ion. It has long been considered that compounds of mercury are diuretics by virtue of the fact that they liberate small quantities of mercuric ion (2). Against this view is the recent evidence (11, 12) that mersalyl and meralluride are excreted either as cysteine or acetyl-cysteine complexes of the parent compound and that little or no inorganic mercuric ion can be found in the urine. Furthermore, it is evident in the data presented in this paper, that the diuretic activity of chlormerodrin and possibly of meralluride exceeds that of mercuric chloride per mg. of mercury administered. It is difficult to believe that chlormerodrin could exert its marked diuretic effect by liberating a minute fraction of its mercury as mercuric ion. A more acceptable explanation of our findings would be that the bonding of mercury to an active enzyme site is facilitated by a hydrophilic group three carbons distant without the necessity of postulating secondary bonding to the enzyme through that hydrophilic group.

diuretic activity, for we have been unable to synthesize and test such compounds. Furthermore, certain compounds (13), most notably Novasurol, have been assigned structures which are in opposition to this thesis. In general, however, the structures of these compounds have not been well established; possibly they constitute no contradiction. Furthermore our criteria of diuresis are more restrictive than those applied by others. Compounds which others have claimed to be diuretics, might in our hands be devoid of diuretic properties. It is perhaps best to consider our thesis merely as a working hypothesis and to admit the possibility that other structures might well be consonant with diuretic activity.

SUMMARY

Twelve organic compounds of mercury and mercuric chloride have been studied with respect to diuretic properties, effects on renal function, regression of plasma concentration and tissue distribution in acute experiments on dogs. Each compound was administered intravenously in doses of 1, 2 and 4 mg. of mercury per kilo of body weight.

Six of the 13 compounds exhibited diuretic properties, seven did not. All compounds were accumulated in the kidneys and no relation between pattern of distribution or renal concentration and diuretic activity could be established. Those compounds which were slowly excreted in the urine exhibited no diuretic activity.

Three of the compounds most widely used as inhibitors of sulfhydryl enzymes *in vitro* were devoid of diuretic properties. If diuresis is related to inhibition of sulfhydryl enzymes, the inhibitors must obviously have properties different from those exhibited by the compounds studied.

Our studies and those of Weiner and Müller (11) and others (12) have led us to the view that diuretic activity of organic compounds of mercury is dependent on some fundamental structural configuration. Within the limits of our experience, namely 12 organic compounds of mercury, 5 of which were diuretics and 7 of which were not, the structure associated with diuretic activity includes (a) a chain of not less than 3 carbon atoms, (b) an atom of mercury attached to the terminal carbon of this chain and (c) some hydrophilic group not less than three carbons distant from the mercury. Probably other structures are compatible with diuretic activity; however, many are not.

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# THE EFFECTS OF PHARMACOLOGIC AGENTS ON THE PULMONARY CIRCULATION IN THE DOG. STUDIES ON EPINEPHRINE, NOR-EPINEPHRINE, 5-HYDROXYTRYPTAMINE, ACETYLCHOLINE, HISTAMINE AND AMINOPHYLLINE [1]

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Despite a large body of literature dealing with the subject of drug-induced pulmonary vasomotor activity, there are divergent opinions as to the significance of such effects. While most investigators agree that the tone of the pulmonary vessels can be changed by drugs, the physiologic importance of such changes has been challenged.

Some of the existing disagreement is probably due to the technical problems of measuring the principal variables in pulmonary hemodynamics, namely the effective pulmonary artery and left atrial pressures, cardiac output and transpulmonary pressure (*i.e.*, the pressure difference between the alveolar and the pleural surfaces of the lung). Further confusion arises from lack of appreciation of the influence of these variables on pulmonary vascular resistance. It is clear from the studies of Edwards (1) and others (2, 3) that these factors have to be rigidly controlled if one is to differentiate direct effects of drugs on the lung vessels from secondary mechanical effects.

In the present study, control of the flow and pressures in the pulmonary circulation in the living dog was accomplished by replacing the right ventricle with a pump and perfusing each lung separately. Blood flow to each lung was measured continuously (2). Drugs were administered into the pulmonary artery of one lung, while the other lung served as a control.

## METHOD

Sixteen dogs weighing between 14 and 29 kg. were anesthetized with either morphine (1.6 mg. per kg.), chloralose and urethane (67 and 670 mg. per kg.) or Nembutal® (34 mg. per kg.) as initial doses.

The animals were tracheotomized and a tracheal divider cannula was inserted. The lungs were ventilated separately by a pair of Starling pumps which gave constant-volume strokes. The chest was opened by sagittal sternotomy and the end-expiratory pressure was adjusted to 5 cm. $H_2O$ in order to limit lung collapse.

The right ventricle was then replaced by a pump circuit and the pulmonary arteries cannulated as described in a previous communication (2). The procedure involved insertion into right atrium and ventricle of an outflow tube which drained systemic venous blood into a reservoir. From there the blood was pumped through flowmeters into the lungs via a pair of cannulae tied into the main pulmonary artery and the left pulmonary artery, respectively. This procedure was performed without interruption of the circulation. Donor blood and dextran [4] were used for priming the extracorporeal circuit. Mepesulfate [5] was used as an anticoagulant.

Flow to each lung was measured with a Shipley-Wilson rotameter. Pressures in both pulmonary arteries beyond the cannulae, in the left atrium, and in the femoral artery were measured with electromanometers. The left and right airway pressures were measured in some experiments with inductance manometers. All values were continuously recorded on an oscillograph. Vascular pressures and flows were expressed as mean values (electrically integrated).

Drug injections and infusions usually were administered into the right and left pulmonary inflow tubes. Administration at a point proximal to the rotameters ensured adequate mixing. All drugs were administered at a fixed pump output (constant total pulmonary flow). In most experiments no attempt was made to prevent recirculation of the drug. This was done because, while the lung receiving the drug obtained a relatively undiluted concentration, any recirculating diluted drug would affect both lungs to an equal degree. Further, the reservoir-pump system ensured more dilution and longer circulation time than would occur in the uncannulated animal.

Unilateral vasomotor activity in this preparation was

[1] Supported by grants from the Life Insurance Medical Research Fund, New York, New York, and the National Heart Institute, U. S. Public Health Service, Bethesda, Maryland.

[2] Present address: Chirurgischen Universitäts-Klinik, Marburg/Lahn, Germany.

[3] Eli Lilly Research Fellow (South Africa).

[4] Expandex, generously supplied through Commercial Solvents Corp., Dr. J. Martin.

[5] Mepesulfate, generously supplied through Hoffman-La Roche, Inc., Dr. E. Severinghaus.

denoted by a redistribution of the blood flow to the two lungs. When this flow redistribution was marked, there was a noticeable deviation from each other of the two pulmonary artery pressures (measured beyond the cannulae). This pressure deviation was caused by the apparatus resistances interposed between the pump and the two pulmonary arteries. A consequence of this artificial pressure deviation was that the actual flow redistribution was somewhat less than would have occurred if the pressures had remained equal. At a blood flow of 0.5 to 1.5 L. per min. through each lung the resistances of the right and left flowmeter cannula systems were 8 to 10 cm. $H_2O$ per L. per min. and 9.5 to 12.5 cm. $H_2O$ per L. per min., respectively.

## RESULTS

### *Epinephrine and nor-epinephrine* [6]

*Epinephrine* (Suprarenin® Bitartrate, Winthrop-Stearns), was rapidly injected into one lung 32 times in 9 experimental preparations. In thirty instances, the injection was followed by a redistribution of the blood flow, the flow decreasing in the test lung. This denoted vasoconstriction. The flows started to change within 6 seconds after the injection, reached their maximum redistribution within one-half minute and returned to control levels within 2 to 3 minutes. The dosage ranged from 0.17 to 6.3 gamma per kg. body weight with an average of 2.3 gamma per kg. The unilateral flow decrease amounted to 4 to 15 per cent (average, 8.6 per cent). For reasons stated above, the flow redistribution is somewhat less than would occur in the absence of the flow metering system. The effect of a given dose was usually more marked on the left because the concentration obtained was higher in the smaller lung. Failure to produce vasoconstriction occurred in two instances and was apparently due to inadequate dosage (2 runs, 0.32 and 0.44 gamma per kg.), for larger doses produced vasoconstriction in the same animals. In different animals there was a marked variability in response.

The effects of epinephrine on pulmonary arterial, left atrial, and femoral arterial pressures and on flow distributions are shown in Table I. In spite of the pulmonary vasoconstriction, the pulmonary artery pressure fell in 13 instances in which left atrial pressure also decreased. Femoral artery pressure rose in 31 of 32 runs. Control femoral artery pressures varied between 70 and 140 mm. Hg.

Airway pressure was unchanged by epinephrine in 5 dogs in which it was measured.

*Nor-epinephrine* (Levophed® Bitartrate, Winthrop-Stearns) was administered to 4 animals on 24 occasions. In 22 instances there was a decrease in blood flow in the lung receiving the drug, denoting pulmonary vasoconstriction. The dosage varied from 0.17 to 6.3 gamma per kg. body weight (average, 2.2 gamma per kg.). The per cent decrease of unilateral flow ranged from 3 to 16 with an average of 7.3 per cent. In two instances (in one dog) pulmonary vasoconstric-

TABLE I

*Effect of epinephrine, injected into one lung, on the blood flow and arterial pressure (PA) of the same lung and on pressures in left atrium (LA) and femoral artery (FA) in 9 dogs—Dose range 0.17 to 6.3 gamma per kg. body weight—The total blood flow was held constant*

| | | Number of observations | Change | |
|---|---|---|---|---|
| | | | Range | Mean |
| Unilat. flow | No change | 2 | | |
| | Fall | 30 | 4–15% | 8.6% |
| PA cm. $H_2O$ | Rise | 17 | 0.2–3.2 | 1.5 |
| | No change | 1 | | |
| | Fall | 14 | 0.3–3.7 | 1.4 |
| LA cm. $H_2O$ | Rise | 5 | 0.7–3.8 | 1.3 |
| | No change | 2 | | |
| | Fall | 25 | 0.2–7.2 | 2.3 |
| FA | Rise | 31 | 3–86% | 30% |
| | Fall | 1 | | 24% |

TABLE II

*Effect of nor-epinephrine, injected into one lung, on the blood flow and arterial pressure (PA) in the same lung and on pressures in left atrium (LA) and femoral artery (FA) in 4 dogs—Dose range 0.17 to 6.3 gamma per kg. body weight —The total blood flow was held constant*

| | | Number of observations | Change | |
|---|---|---|---|---|
| | | | Range | Mean |
| Unilat. flow | No change | 2 | | |
| | Fall | 22 | 3–16% | 7.3% |
| PA cm. $H_2O$ | Rise | 11 | 0.4–2.6 | 1.7 |
| | No change | 1 | | |
| | Fall | 12 | 0.3–1.4 | 0.6 |
| LA cm. $H_2O$ | Rise | 3 | 0.5–1.5 | 1.1 |
| | Fall | 21 | 0.6–6.0 | 2.8 |
| FA | Rise | 24 | 6–90% | 44% |

[6] Dosage expressed in epinephrine and nor-epinephrine base.

TABLE III

*Effect of serotonin infusion into one lung—Total blood flow kept constant—Both PA pressures equal*

| Dog No. | Side | No. of steps | Duration (*min.*) per step | Dose *gamma/kg./min.* | Max. % decr. of unilateral flow |
|---|---|---|---|---|---|
| 55 | Lt. | 1 | 4 | 20 | 45 |
| 55 | Rt. | 1 | 2.5 | 20 | 29 |
| 63 | Rt. | 5 | 2 | 12–33.6 | 55 |
| 63 | Lt. | 3 | 2.5 | 12–33.6 | 79 |
| 63 | Rt. | 3 | 3 | 12–19.2 | 47 |
| 63 | Rt. | 3 | 2 | 12–33.6 | 43 |

tion failed to occur after 0.5 gamma per kg.; in this experiment vasoconstriction was produced by larger doses. The response was rapid. There was also a marked variation of responsiveness in different animals.

As with epinephrine administration, the pulmonary artery pressure tended to follow left atrial pressure change (Table II). In 10 instances in which left atrial pressure fell, pulmonary arterial pressure also decreased in spite of the pulmonary vasoconstriction. Femoral artery pressure rose by an average of 44 per cent (range, 6 to 90 per cent) from control values of 70 to 122 mm. Hg.

### *5-Hydroxytryptamine*

5-Hydroxytryptamine (Serotonin Creatinin Sulfate—Sandoz)[7] was administered into one pulmonary artery in 5 dogs. There were 2 injections (2.3 and 4.5 gamma per kg. body weight) and 11 infusions (2.3 to 33.6 gamma per kg. body weight per min.). In five of the infusions the dosage was increased in steps.

All doses resulted in marked pulmonary vasoconstriction in the lung receiving the drug, denoted by large redistribution of blood flow and a marked rise in both pulmonary artery pressures. There was also a noticeable discrepancy between the right and left pulmonary artery pressures, as explained under methods. In 2 animals the pulmonary artery pressures were made equal at the height of the response by constricting the respective pulmonary inflow tubes; the resulting decrease in blood flow on the side receiving the infusion is shown in Table III. One of these experiments is illustrated in Figure 1.

[7] Dosage expressed as Serotinin creatinin sulfate.

The onset of pulmonary vasoconstriction occurred within a few seconds at any given dose. The duration of action after stopping the infusions varied between 5 and 15 minutes, depending on the dosage and the duration of infusion. Pulmonary artery pressures increased in all runs, irrespective of coincident changes in left atrial pressure. Left atrial pressure followed directionally the changes in femoral artery pressure.

Mean femoral artery pressure did not behave in a consistent pattern. It usually was unchanged unless very high doses were administered when it showed large fluctuations around the control value. No bronchoconstriction was produced in these experiments.

### *Acetylcholine and Histamine*

Acetylcholine, given as an aerosol into one lung, produced an increase in airway pressure. Since

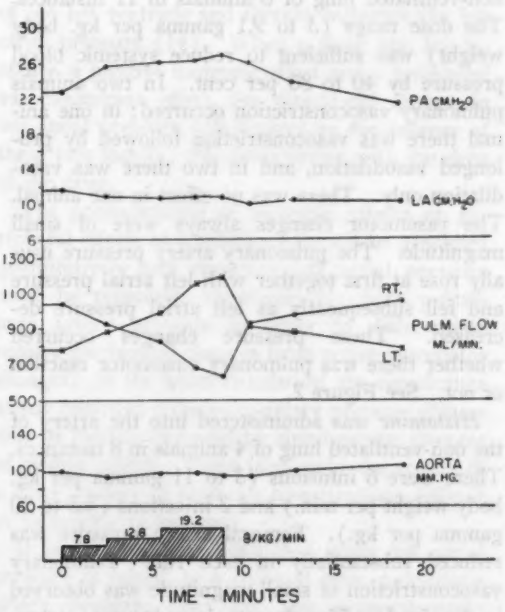


FIG. 1. EXP. NO. 63. BODY WEIGHT 21 KG. EFFECT OF 5-HYDROXYTRYPTAMINE, INFUSED INTO THE RIGHT PULMONARY ARTERY, ON PULMONARY VASCULAR PRESSURES (PA AND LA), LEFT AND RIGHT LUNG BLOOD FLOW AND AORTIC PRESSURE

Total pulmonary blood flow was held constant. Vasoconstriction in the treated lung is denoted by the altered flow distribution. The pressures in the two pulmonary arteries were made equal by constriction of the left inflow tubing.

the lung was ventilated by a constant-volume pump, this was presumably due to bronchoconstriction. The increase of airway pressure resulted in a decrease of blood flow through the lung under study with a corresponding increase in flow on the other side. In these experiments the inspiratory airway pressure rose 4 to 10 cm. $H_2O$ and the flow in the tested lung decreased 10 to 20 per cent.

Acetylcholine and histamine also produced a rise in airway pressure when administered into the pulmonary artery. Experiments were therefore performed in which drugs were given to the collapsed or air-free lung. A lung was made air free by ventilating the dog with pure oxygen and then allowing the gas to be absorbed from the collapsed lung. Induced bronchoconstriction then had no effect on intra-alveolar pressure.

*Acetylcholine* was injected into the artery of the non-ventilated lung of 6 animals in 11 instances. The dose range (3 to 9.1 gamma per kg. body weight) was sufficient to reduce systemic blood pressure by 40 to 80 per cent. In two animals pulmonary vasoconstriction occurred; in one animal there was vasoconstriction followed by prolonged vasodilation, and in two there was vasodilation only. There was no effect in one animal. The vasomotor changes always were of small magnitude. The pulmonary artery pressure usually rose at first together with left atrial pressure and fell subsequently as left atrial pressure decreased. These pressure changes occurred whether there was pulmonary vasomotor reaction or not. See Figure 2.

*Histamine* was administered into the artery of the non-ventilated lung of 4 animals in 8 instances. There were 6 infusions (3 to 11 gamma per kg. body weight per min.) and 2 injections (4.3 to 20 gamma per kg.). Femoral artery pressure was reduced substantially in each run. Pulmonary vasoconstriction of small magnitude was observed in 3 animals. The changes in pulmonary artery pressure again reflected the changes in left atrial pressure.

### *Aminophylline*

Aminophylline was injected in doses ranging from 2.2 to 24 mg. per kg. body weight to 5 animals in 8 instances. Pulmonary vasodilation was produced in 3 instances in 2 animals (dosage, 2.4 to 2.9 mg. per kg. body weight, unilateral increase of flow, 7 to 14 per cent). In each run, systemic blood pressure and left atrial pressure were reduced substantially. Pulmonary artery pressure fell concomitantly with left atrial pressure.

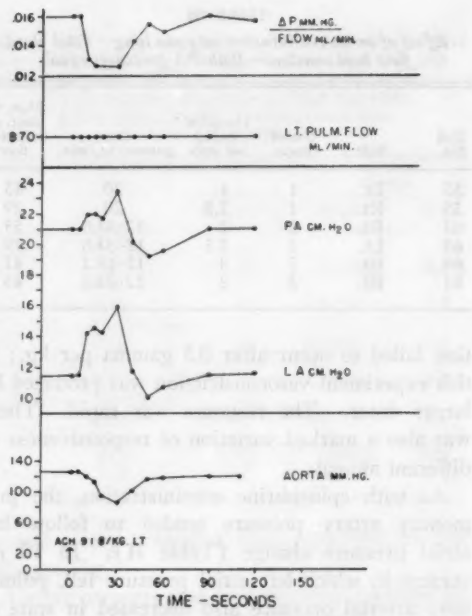


FIG. 2. EXP. NO. 64. BODY WEIGHT 22 KG. EFFECT OF ACETYLCHOLINE, INJECTED INTO LEFT PULMONARY ARTERY

Total pulmonary flow was held constant; there was no change of left pulmonary flow, indicating no pulmonary vasomotion. Note the changes in pulmonary vascular pressures (PA and LA) and left pulmonary vascular resistance in spite of the absence of pulmonary vasomotion and flow change.

## DISCUSSION

Sharpey Schafer and Lim (4) and Friedberg, Katz, and Steinitz (5) have drawn attention to the complex factors which determine the response of the lesser circulation to drugs. In addition to direct effects on the tone of the pulmonary vessels, there are secondary mechanical changes of pulmonary vascular geometry and resistance due to changes in cardiac output, left atrial pressure, and transpulmonary pressure. The qualitative and quantitative effects of these factors on pulmonary vascular resistance have been studied separately (2, 3, 6).

The preparation used for these experiments facilitates the evaluation of pulmonary vasomotor tone changes. The drug is injected into one lung while the other lung is used as the control. Redistribution of flow is used as the criterion for vasomotor activity instead of changes in pulmonary arterial pressure and resistance. Alterations of vascular resistance due to changes of pulmonary vasomotor tone can be differentiated from changes of resistance due to left atrial pressure variation, since the latter pressure influences both pulmonary vascular beds. Alterations of pulmonary vascular resistance due to changes in transpulmonary pressure can be avoided. A comparison of pulmonary and peripheral vascular effects is also possible.

*Vasomotor changes*

Epinephrine and nor-epinephrine were found to have a consistent but moderate vasoconstrictor effect. A comparison of the pulmonary and systemic actions of these drugs gives an indication of the probable physiologic importance of their pulmonary vasomotor effects. Whereas the femoral artery pressure responses to both drugs were marked, the changes in pulmonary arterial pressure were of only the same magnitude as those of the left atrial pressure. The blood flow redistribution in the two lungs was relatively small even with the large doses used. A marked cardiac inotropic effect is produced by less than 1 gamma per kg. of these drugs (7). It is concluded that the action of these drugs on pulmonary vascular tone is of small magnitude and of minor hemodynamic importance. Results in agreement with these were obtained by Edwards (1) and Rose, Freis, Hufnagel, and Massullo (8) in experiments in which flow and pulmonary venous pressures were controlled.

On the other hand, serotonin is a potent pulmonary vasoconstrictor agent, as is suggested by other studies (9, 10). The systemic circulation was affected only when large doses of serotonin were infused, or when serotonin was administered intra-arterially. Marked pulmonary vasoconstriction with, indeed, systemic vasodilation was found also by Rudolph and Paul (11). The prominent pulmonary effect of serotonin is of interest in view of the fact that it is liberated when intravascular clotting occurs and thus may play a role in the vascular response to pulmonary embolism.

The lack of consistent pulmonary vasodilation with drugs like acetylcholine and Aminophylline may be due to the fact that the "control" tone of the pulmonary vascular bed was low. Apparently these drugs may cause a marked vasodilation in patients with increased pulmonary vascular tone (12, 13).

*Changes in pulmonary vascular resistance due to changes in left atrial pressure*

Left atrial pressure (left ventricular filling pressure) may be altered by drugs in two ways in this preparation in which the ventricular output is kept constant. A positive inotropic drug will lower the left atrial pressure; a negative inotropic drug will raise it. On the other hand, systemic vasomotor action of a drug will alter the work load of the left ventricle and thereby influence the left atrial pressure (14). When a drug has, for instance, a positive inotropic effect and a peripheral vasoconstrictor effect, the directional change in left atrial pressure will depend on the balance of these two factors; this often varies with the dose of the drug.

The predominance of inotropic effects or peripheral effects of a substance cannot be predicted quantitatively and therefore left atrial pressure must be measured simultaneously with pulmonary artery pressure in order to differentiate passive mechanical changes from active vasomotor changes. In the intact circulation, the significance of any recorded pulmonary artery pressure change may be further obscured by simultaneous changes of cardiac output. These facts make the results of a large number of previous investigations difficult or impossible to interpret.

It is important to recall that when left atrial pressure is raised, at a constant flow, pulmonary artery pressure does not rise proportionately (Figure 2). Equal rises in both pressures occur only at high degrees of pulmonary vascular distension, when resistance does not decrease any further (2, 3). For this reason the measurement of the pressure drop from the pulmonary artery to the left atrium alone (15) is not sufficient for assessing active pulmonary vasomotion.

Pulmonary vascular resistance, *i.e.*, the pressure drop from the pulmonary artery to the left atrium

divided by flow rate is inversely related to the fourth power of the radii of all vessels contributing to resistance. It has been shown that in the highly distensible pulmonary vascular bed small changes of the distending pressures (both the pulmonary arterial and left atrial pressures) can produce large changes in resistance (2, 3). When left atrial pressure rises or flow increases, resistance falls.

The changes in pulmonary resistance produced by substances which affect predominantly the heart and systemic circulation frequently reflect the mechanical behavior of the lung vessels rather than active pulmonary vasomotion (Figure 2). On the other hand, the pulmonary resistance changes induced by serotonin, for example, are due to active pulmonary vasomotion rather than indirect mechanical effects.

*Effect of airway pressure*

The caliber of vessels which contribute to pulmonary vascular resistance is affected by changes in transpulmonary pressure, *i.e.*, the pressure difference between the alveolar and pleural surfaces of the lung. When flow is kept constant, resistance to flow rises when the lung is inflated (6). It is thought that the capillaries are compressed by inflation of the lung.

In these experiments, histamine and acetylcholine both increased transpulmonary pressure by way of bronchoconstriction and when this was marked, resistance to flow increased. Similar conclusions could be drawn from the findings of Field and Drinker (16), von Euler (17) and Müller, Salomon, and Zuelzer (18). When the effects of bronchoconstriction on the transpulmonary pressure had been abolished by collapsing the lung, a consistent vasoconstrictor response to these drugs could not be demonstrated. It is necessary, therefore, to consider a possible change in transpulmonary pressure when pulmonary vasomotion is being investigated.

SUMMARY

1. A preparation was used which permitted the differentiation of active pulmonary vasomotor responses from secondary mechanical changes in the lung vessels. Unilateral pharmacologically induced pulmonary vasomotion was studied with a number of drugs.

2. Epinephrine, nor-epinephrine and 5-hydroxytryptamine produced consistent pulmonary vasoconstriction. Of the drugs studied, 5-hydroxytryptamine had the most marked pulmonary vasomotor effect.

3. Acetylcholine and histamine increased transpulmonary pressure. When this effect was prevented, inconsistent pulmonary vasomotor effects of small magnitude were found.

4. Aminophylline produced pulmonary vasodilation in some animals while it had no effect in others.

5. The prerequisites for the assessment of active pulmonary vasomotor changes in the lungs have been discussed.

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# THE DISTRIBUTION OF $Fe^{59}$ TAGGED HUMAN ERYTHROCYTES IN CENTRIFUGED SPECIMENS AS A FUNCTION OF CELL AGE

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This study demonstrates that the density of human erythrocytes increases with age and that cell populations with different mean ages can be isolated on the basis of this density gradient. The data suggest that the concentration of $Fe^{59}$ in the bottom layer of centrifuged erythrocyte specimens may be used to estimate the mean life span of ageing cells.

## METHODS

Six to ten microcuries of $Fe^{59}$ were administered intravenously to eight hematologically normal subjects who had various chronic diseases (Table I). Five of the subjects received $Fe^{59}$ iron globulin complex [1] and three received $Fe^{59}$ ferrous citrate [1] that had been incubated with the subject's plasma or whole blood before injection. Specific activity of both preparations was 0.5 to 1.5 mC. per mgm. at the time of administration. One subject (C.A.) [2] also received 100 $\mu$C. of $Cr^{51}$ (1).

Twenty ml. heparinized blood specimens were drawn at the intervals indicated in Figure 1 and centrifuged in 10-ml. plastic tubes for 40 minutes at 3,000 RPM (approximately 2,000 G.). The tubes were placed in an alcohol-dry ice freezing mixture for a few minutes and were then cut 1 to 2 mm. below the plasma-erythrocyte interface. The bottom portions containing the frozen erythrocytes were cut into four equal sections. The radioactivity of thawed aliquots from each section was measured in a well-type scintillation counter with a Tl-activated NaI crystal and expressed as counts per second per ml. of erythrocytes. The $Fe^{59}$ and $Cr^{51}$ activity in specimens from C.A. were measured separately by differential pulse height analysis (1). Correction for isotope decay was based on the radioactivity of standards which were prepared from the injected material and counted with each blood specimen. The standard error of the counting procedure was less than $\pm 2$ per cent for most specimens and less than $\pm 4$ per cent for the low activity specimens.

The proportion of erythrocytes discarded with the plasma ($8 \pm 2$ per cent) [3] was calculated from the total hemoglobin in plasma and erythrocyte sections of fourteen specimens.

$Fe^{59}$ activity in unseparated whole blood erythrocytes was determined in twenty specimens from five subjects by counting aliquots of whole blood and dividing by the aliquot volume and hematocrit.

## RESULTS

$Fe^{59}$ concentration was highest in the top layer and lowest in the bottom layer of erythrocyte specimens drawn during the first 15 days after administration of the isotope. The concentration decreased in the top layer and increased in the bottom layer of specimens drawn between the 15th and 90th days, and this trend reversed between the 90th and 150th days (Figure 1). The extremes of individual variation are illustrated by subjects S.I. and R.I. The systematic nature of the changes

TABLE I

*Clinical data*

| Subject | Sex | Age | Clinical diagnosis |
|---|---|---|---|
| C. A. | F | 54 | Hypertensive cardiovascular disease. Cerebral vascular accident. |
| C. E. | M | 66 | Hypertensive cardiovascular disease. Cerebral arteriosclerosis. |
| S. I. | M | 64 | Stasis dermatitis. Arteriosclerotic heart disease. |
| A. R. | M | 56 | Arteriosclerotic heart disease. Osteoarthritis of the spine. |
| G. E. | M | 62 | Rheumatic heart disease. |
| B. U. | M | 59 | Hypertensive cardiovascular disease. Arteriolar nephrosclerosis. Cerebral vascular accident. |
| R. I. | M | 58 | Peripheral neuritis. Cerebellar degeneration. Duodenal ulcer. |
| O. C. | M | 58 | Hypertensive cardiovascular disease. Cerebral vascular accident. |

[1] $Fe^{59}$ iron "IV–7 globulin" complex and $Fe^{59}$ ferrous citrate were obtained from Abbott Laboratories, Chicago, Illinois.

[2] Subject C. A. and a patient with non-spherocytic hemolytic anemia were studied in co-operation with Dr. I. M. Weinstein. Data from this and other patients with hematological abnormalities will be reported separately.

[3] Data reported in this manner represent the mean and standard deviation.

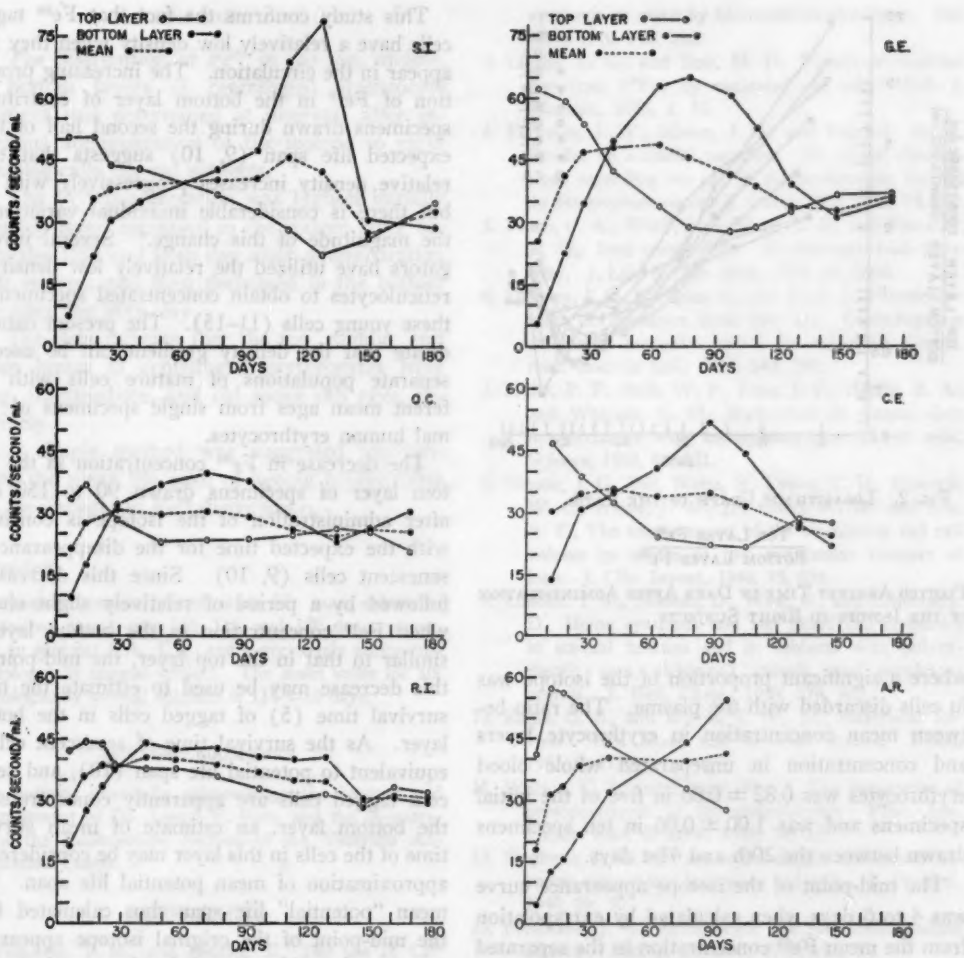


FIG. 1. THE $Fe^{59}$ CONCENTRATION IN THE TOP AND BOTTOM LAYERS OF ERYTHROCYTES FROM CENTRIFUGED BLOOD SPECIMENS PLOTTED AGAINST TIME IN DAYS AFTER ADMINISTRATION OF THE ISOTOPE

The mean $Fe^{59}$ concentration in the four layers of erythrocytes is also plotted against time.

in isotope distribution with time is demonstrated in the logarithmic graph of the ratio

$$\frac{\text{Top layer } Fe^{59}}{\text{Bottom layer } Fe^{59}}$$

(Figure 2). This ratio decreased progressively during the first 80 to 130 days and then increased to a second maximum slightly greater than unity after 130 days.

In almost all specimens the $Fe^{59}$ concentration in the middle layers was intermediate between that in the top and bottom. However, the concentration was slightly higher in the middle layer than in either the top or bottom of specimens from three subjects drawn between the 40th and 55th days, when the ratio $\frac{\text{Top layer } Fe^{59}}{\text{Bottom layer } Fe^{59}}$ was near unity.

The mean $Fe^{59}$ concentration in the separated erythrocyte layers approximated the concentration in the unseparated whole blood erythrocytes of the same specimens, except in the initial specimens

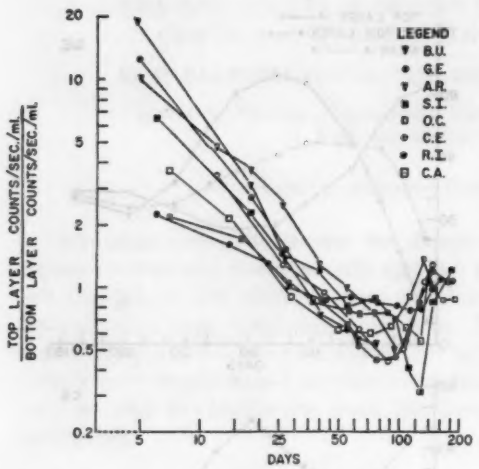


FIG. 2. LOGARITHMIC GRAPH OF THE RATIO

$$\frac{\text{TOP LAYER } Fe^{59}}{\text{BOTTOM LAYER } Fe^{59}}$$

PLOTTED AGAINST TIME IN DAYS AFTER ADMINISTRATION OF THE ISOTOPE TO EIGHT SUBJECTS

where a significant proportion of the isotope was in cells discarded with the plasma. The ratio between mean concentration in erythrocyte layers and concentration in unseparated whole blood erythrocytes was $0.82 \pm 0.06$ in five of the initial specimens and was $1.00 \pm 0.06$ in ten specimens drawn between the 20th and 41st days.

The mid-point of the isotope appearance curve was 4 to 6 days when calculated by extrapolation from the mean $Fe^{59}$ concentration in the separated layers, and was 4 days when calculated from the concentration in whole blood specimens of five subjects.

## DISCUSSION

Previous investigators have shown that $Fe^{59}$ incorporation is limited to immature cells of the erythrocyte series (2, 3) and that $Fe^{59}$ tagged cells which appear in the circulation during the first five days after administration of the isotope to humans or rats have a relatively low density (4–6). Since there is probably no significant iron exchange in mature erythrocytes (7, 8), the change in relative density of $Fe^{59}$ tagged cells may be followed throughout their life span.

This study confirms the fact that $Fe^{59}$ tagged cells have a relatively low density when they first appear in the circulation. The increasing proportion of $Fe^{59}$ in the bottom layer of centrifuged specimens drawn during the second half of their expected life span (9, 10) suggests that their relative density increases progressively with age but there is considerable individual variation in the magnitude of this change.[4] Several investigators have utilized the relatively low density of reticulocytes to obtain concentrated specimens of these young cells (11–15). The present data indicate that the density gradient can be used to separate populations of mature cells with different mean ages from single specimens of normal human erythrocytes.

The decrease in $Fe^{59}$ concentration in the bottom layer of specimens drawn 90 to 150 days after administration of the isotope is consistent with the expected time for the disappearance of senescent cells (9, 10). Since this decrease is followed by a period of relatively slight change when $Fe^{59}$ concentration in the bottom layer is similar to that in the top layer, the mid-point of this decrease may be used to estimate the mean survival time (5) of tagged cells in the bottom layer. As the survival time of senescent cells is equivalent to potential life span (10), and senescent tagged cells are apparently concentrated in the bottom layer, an estimate of mean survival time of the cells in this layer may be considered an approximation of mean potential life span. The mean "potential" life span thus calculated from the mid-point of the original isotope appearance curve to the mid-point of the decrease in isotope concentration in the bottom layer was 103, 119, and 134 days, respectively, in subjects O.C., G.E., and S.I. One patient with non-spherocytic hemolytic anemia had a shortened "potential" life span of 52 days as well as a shortened $Cr^{51}$ half-life survival time of 16 days.[2]

[4] Individual variations may be related to the magnitude of the gradient in density between cell populations of different ages or to other physical factors influencing their relative separability by centrifugation. Data from unpublished experiments demonstrate that the difference in density between the top and bottom layers of erythrocytes separated by this method is $8.3 \pm 6.2$ gm. per L. (S.E. 0.92 gm. per L.; $p < 0.001$). Density was calculated from the weight of known volumes corrected for measured trapped plasma.

SUMMARY

1. The distribution of $Fe^{59}$ in the top, middle, and bottom layers of centrifuged erythrocyte specimens was determined at intervals after intravenous administration of the isotope to eight hematologically normal subjects.

2. The $Fe^{59}$ concentration was highest initially in the top layer, increased in the bottom layer between the 15th and 90th day after administration of the isotope, and then decreased in the bottom layer after the 90th day.

3. The data indicate that the top, middle, and bottom layers of centrifuged erythrocytes have young, intermediate, and old mean cell ages, respectively.

4. A simple method was suggested for estimating the mean life span of ageing erythrocytes from the changes in $Fe^{59}$ distribution.

### *Addendum*

The mean "potential" erythrocyte life span, calculated as described above, was 115, 115, and 120 days, respectively, in subjects C.A., C.E., and a previously unreported hematologically normal subject. The mean value for six hematologically normal subjects is $118 \pm 10$ days.

ACKNOWLEDGMENTS

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# STRONTIUM$^{85}$ AND CALCIUM$^{45}$ METABOLISM IN MAN [1, 2]

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Preliminary reports were recently published from this laboratory on the metabolism of intravenously administered radiostrontium and on the effect of calcium upon strontium excretion in man (1, 2). The absorption, the digestive juice excretion and tissue retention have been studied following the oral and intravenous administration of $Sr^{85}$. Furthermore, the metabolism of orally administered radiostrontium has been compared with that of radiocalcium in the same patients (3, 4). These data form the basis of this communication.

## MATERIALS AND METHODS

The six patients listed in Table I were studied on the Metabolic Research Ward. They were maintained on a controlled, analyzed, low calcium diet containing approximately 150 mg. calcium and 600 mg. phosphorus per day (5). Patient 4 received 520 mg. calcium as daily supplement in the form of calcium gluconate. The diet as well as the excreta were analyzed for nitrogen, calcium and phosphorus and the metabolic balances of these constituents were determined on six-day metabolic pools. The urinary calcium was determined daily, the serum calcium once weekly. At the start of each study the patients received at 8:30 a.m. a tracer dose (0.1 to 0.4 $\mu$c. per kg.) [4] of $Sr^{85}$ as the chloride. The radioactive solution was administered *orally* with breakfast to insure proper mixing of the isotope with the food. At the conclusion of these studies which lasted 12 to 40 days a second tracer of $Sr^{85}$ was administered *intravenously* to four of these patients in the fasting state. $Sr^{85}$ chloride (0.1 to 0.4 $\mu$c. per kg.) was injected rapidly into the clamped distal portion of the tubing of an intravenous infusion set. After completion of the injection the clamp was released and the isotope solution was flushed rapidly into the vein with the infusion fluid of 5 per cent glucose in water. The intravenous studies lasted 14 to 16 days.

$Sr^{85}$ is a carrier-free isotope [5] produced by bombardment of rubidium$^{85}$. It is a pure $\gamma$-emitter, its energy is 0.51 mev. and its half-life is 65 days. The radioactivity of duplicate samples of plasma, urine and stool was measured in a well-type scintillation counter. The stability of the counting arrangement was checked daily against a uranium standard. The statistical counting error varied: in the initial phases of the experiment sufficient counts were taken to keep the probable error within 2 per cent. In later phases, counting rates were lower, and it was not feasible to maintain this precision, so that counting errors as high as 5 per cent were incurred. Standards were prepared for each patient at the time of administration of the isotope by delivering the same dose into a volumetric flask; appropriate dilutions were then prepared for counting. The standards were counted along with each set of experimental samples, thereby correcting for the decay of the isotope.

The radioactivity was determined on 2 to 5 ml. of plasma obtained 5, 15 and 30 minutes and 1, 4, 8 and 24 hours after the ingestion or injection of the isotope. Thereafter, plasma was obtained every morning in the post-absorptive state during the first week of each study and 2 to 3 times per week thereafter. The plasma radioactivity was calculated for the total plasma volume which was determined for each patient using T-1824 dye (6). The urinary output was separated on the day of the isotope administration in fractions at 1, 4, 8 and 24 hours. On the subsequent days, the 24-hour urine output was collected. Aliquots of 2 to 5 ml. of urine were used for radio-assays. Each stool specimen was collected separately, water was added to obtain a homogeneous mixture in a Waring blendor. The weight of the homogenate was determined and aliquots of 4 to 5 gm. were assayed in the scintillation counter. Multiple analyses of such aliquots indicated homogeneous distribution of the isotope. Since the radioactivity of the stool specimens was high after the ingestion of the tracer, these specimens were counted in a bismuth cathode $\gamma$-counter. The radioactivity was determined on the total stool homogenate and on several water rinses of the Waring blendor using Marinelli beakers.

Two patients (2 and 6, Table I) also received an oral tracer of $Ca^{45}$ prior to the administration of oral $Sr^{85}$. Radiocalcium was administered orally as the chloride

[1] This study was supported by the U. S. Atomic Energy Commission, Contract AT(30-1)-1763, by grants from the National Cancer Institute (C-1540) and National Institute of Arthritis and Metabolic Diseases (A-855), National Institutes of Health, United States Public Health Service.

[2] Presented at the 76th Meeting of the American Physiological Society, Federation of American Societies for Experimental Biology, Atlantic City, April, 1956.

[3] Trainee, National Cancer Institute.

[4] A higher tracer dose was given to the first two patients of this study (Patients 5 and 6, Table I), to insure sufficient counting accuracy of the specimens for approximately 40 days.

[5] Produced by the Nuclear Science and Engineering Corporation, Pittsburgh, Pa.

TABLE I

*Patients receiving $Sr^{85}$ orally and intravenously*

| Patient number | Age | Sex | Diagnosis | Route of $Sr^{85}$ administration | Duration of study *days* |
|---|---|---|---|---|---|
| 1 H. R. | 58 | M | Carcinoma of lung. No bone metastases. Partial immobilization. | Oral<br>Intravenous | 13<br>15 |
| 2 I. B. | 54 | F | Carcinoma of uterus; lymphoma; osteoporosis. No bone metastases. | Oral<br>Intravenous | 12<br>14 |
| 3 B. H. | 76 | F | Senile osteoporosis | Oral<br>Intravenous | 14<br>16 |
| 4 H. B. | 63 | M | Paget's disease of bone | Oral<br>Intravenous | 12<br>14 |
| 5 S. M. | 55 | F | Carcinoma of breast. Bone metastases. | Oral | 40 |
| 6 B. L. | 72 | M | Carcinoma of larynx. No bone metastases. | Oral | 40 |

in isotonic saline with breakfast. The plasma levels, urinary and fecal excretions of $Ca^{45}$ were followed in a manner similar to that outlined for $Sr^{85}$. The data on the $Ca^{45}$-metabolism of these two patients who were studied for 74 days and the techniques employed were previously published (7, 8).

## RESULTS

The metabolism of an intravenously administered dose of $Sr^{85}$ (plasma levels, urinary and fecal excretions) was described in a previous publication (2).

The plasma levels and urinary $Sr^{85}$ excretions of two patients in the 24 hours following the oral administration of the tracer are listed in Table II.

Radioactivity was detected within 5 minutes in the plasma of B.L. and within 15 minutes of S.M. The $Sr^{85}$ plasma level increased gradually, was highest 4 hours after ingestion of the tracer and declined thereafter. The rate of urinary excretion of $Sr^{85}$ at 1, 4, 8 and 24 hours as well as the cumulative urinary strontium excretions are listed in Table II. The highest rate of $Sr^{85}$ excretion per hour, 0.24 per cent of the dose, was noted between the 2nd and 4th hour in S.M. while it occurred between the 8th and 24th hour in B.L. at a level of 0.044 per cent of the dose. The cumulative urinary $Sr^{85}$ excretion in the first 24 hours was 2.42 per cent and 0.90 per cent for the two patients, respectively.

TABLE II

*$Sr^{85}$ in plasma and urine following the ingestion of the tracer*

| Time interval after ingestion of $Sr^{85}$ | S. M. Patient 5 | | | B. L. Patient 6 | | |
|---|---|---|---|---|---|---|
| | $Sr^{85}$, % dose | | | $Sr^{85}$, % dose | | |
| | Plasma* | Urine | | Plasma* | Urine | |
| | | Rate/hour | Cumulative | | Rate/hour | Cumulative |
| 5 minutes | 0 | | | 0.035 | | |
| 15 minutes | 0.036 | | | 0.041 | | |
| 30 minutes | 0.081 | | | 0.18 | | |
| 1 hour | 0.48 | 0.04 | 0.04 | 0.28 | 0.002 | 0.002 |
| 2 hours | 1.67 | 0.19 | 0.23 | 0.77 | 0.014 | 0.016 |
| 4 hours | 1.85 | 0.24 | 0.70 | 1.60 | 0.029 | 0.074 |
| 8 hours | 1.26 | 0.11 | 1.13 | 1.16 | 0.030 | 0.194 |
| 24 hours | 0.80 | 0.08 | 2.42 | 0.74 | 0.044 | 0.904 |

* Calculated per total plasma volume. The plasma samples were obtained at the end of the indicated time intervals.

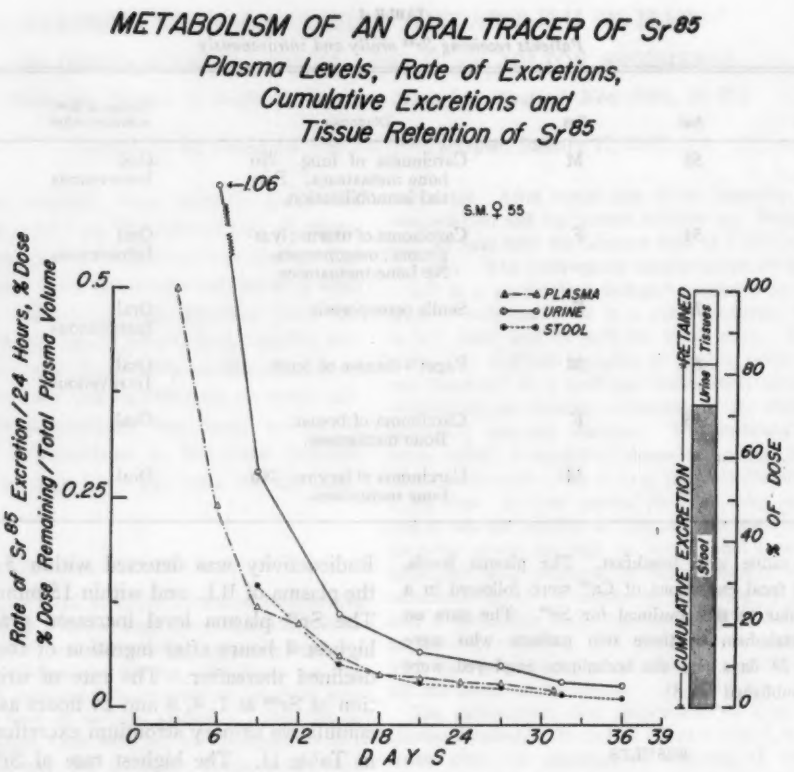


FIG. 1. RATES OF URINARY AND FECAL $Sr^{85}$ EXCRETION PER DAY FOR EACH SIX-DAY METABOLIC PERIOD PLOTTED AT THE MID-POINT

One and six hundredths per cent $Sr^{85}$ was excreted in the urine in the first six days of the study.

Figure 1 illustrates the metabolism of an orally administered tracer dose of $Sr^{85}$ over a period of 39 days (Patient 5, Tables I and II). The plasma levels and rate of urinary $Sr^{85}$ excretions declined after the fourth hour with time. The stool radioactivity was initially very high due to the presence of the unabsorbed dose; 70.7 per cent of the dose was contained in the stool by the fourth day of the study; a total of 71.2 per cent was excreted in six days. These values were too high to be included in the figure. After the passage of the unabsorbed fraction of the dose, the stool radioactivity declined gradually. The rate of urinary $Sr^{85}$ excretion remained higher than the plasma level throughout the entire study. The bar-graph shows the total excretion and tissue retention by 39 days of the study: 72.7 per cent of the dose passed in the stool, 9.5 per cent in the urine. Less than 0.02 per cent remained in the plasma so that only approximately 18 per cent of the dose was retained in the tissues, in contrast to the much higher retention following the intravenous tracer (2).

The results of a 12-day study of 4 patients who received $Sr^{85}$ orally and intravenously are listed in Table III. The urinary excretion differed greatly from patient to patient and ranged from 57.2 to 14.2 per cent of the administered dose after the intravenous injection of the tracer. The excretion was lowest in Patient 4 who had Paget's disease of the bone. The $Sr^{85}$ content of the stool also varied but to a lesser extent, from 14.2 to 6.5 per cent and was lowest in the patient with Paget's disease. The total excretions of $Sr^{85}$ in urine

and stool in 12 days and the percentage of the administered dose retained in the body are listed for each patient in the respective columns. The lower half of Table III lists the results obtained in the same patients following the oral administration of $Sr^{85}$. The cumulative urinary excretions in 12 days were much lower than following the intravenous dose, the lowest, 2.9 per cent, was again noted in Patient 4. An average of 80 per cent of the dose (78.3 to 82.5 per cent) was contained in the stool. Due to the high strontium content of the stool, the total $Sr^{85}$ excretion was higher and the remaining tissue retention considerably lower after the oral than the intravenously administered tracer dose. Approximately four to six times as much $Sr^{85}$ was retained after the intravenous than after the oral dose. The first patient retained only 6.2 per cent of the oral dose after 12 days.

TABLE III

EXCRETION AND RETENTION OF $Sr^{85}$ 12 DAYS FOLLOWING AN I.V. AND ORAL TRACER DOSE

| | INTRAVENOUS $Sr^{85}$ % OF ADMINISTERED DOSE | | | |
|---|---|---|---|---|
| | CASE 1 | CASE 2 | CASE 3 | CASE 4 |
| URINE | 57.2 | 35.3 | 31.6 | 14.2 |
| STOOL | 10.4 | 12.7 | 14.2 | 6.5 |
| TOTAL EXCRETION | 67.6 | 48.0 | 45.8 | 20.7 |
| RETAINED | 32.4 | 52.0 | 54.2 | 79.3 |
| | ORAL | | | |
| URINE | 14.8 | 8.8 | 8.4 | 2.9 |
| STOOL | 79.0 | 82.5 | 78.3 | 81.5 |
| TOTAL EXCRETION | 93.8 | 91.3 | 86.7 | 84.4 |
| RETAINED | 6.2 | 8.7 | 13.3 | 15.6 |

*Calculation of endogenous fecal strontium*

Following the intravenous dose, the stool radioactivity is of endogenous origin, *i.e.*, $Sr^{85}$ excreted with the digestive juices into the gastrointestinal tract minus the fraction which is reabsorbed. However, following the *oral* dose, the radioactivity of the stool is of exogenous and of endogenous origin, the former being the unabsorbed $Sr^{85}$. Knowing the endogenous fecal strontium from the intravenous dose, the exogenous or unabsorbed fraction of the total stool radioactivity following the *oral* dose can be calculated. In this calculation it is assumed that the pattern of excretion of strontium with the digestive juices is the same whether the tracer is injected intravenously or absorbed from the gastrointestinal tract. The validity of this assumption has been proven for radiocalcium in studies performed on animals (9, 10) and in man (11).

*Example.* Twelve and seven tenths per cent of the intravenously administered dose of $Sr^{85}$ was excreted in the stool of Patient 2 (Table III) *i.e.*, endogenous fecal strontium. Let x be the percentage of the oral dose which was absorbed and y the remainder which passes unabsorbed. Following the oral dose the total stool strontium of this patient was 82.5 per cent, consisting of this unabsorbed fraction y plus the endogenous fecal strontium which is 12.7 per cent of the absorbed fraction x, or 0.127 x. Therefore,

$$x + y = 100\%$$

Therefore

$$0.127x + y = 82.5$$
$$x = 20$$
$$y = 80$$

It follows that of the 82.5 per cent radioactivity contained in the stool 80 per cent is the unabsorbed tracer while the endogenous fecal strontium is 2.5 per cent, and 20 per cent is the absorbed dose. The other three patients absorbed 23.4, 25.4, and 19.8 per cent of the administered dose, respectively.

The endogenous fecal strontium of the 4 patients ranged from 1.3 to 3.5 per cent (Table IV). The lowest value was obtained in the patient with Paget's disease of the bone.

*Comparison of the metabolism of intravenously and orally administered $Sr^{85}$*

The data are summarized for 12 days and are listed in Table IV. As shown in Column 2, 25.4 to 19.8 per cent of the oral dose was absorbed. The total excretion—urinary and endogenous fecal strontium—ranged from 17.2 to 4.2 per cent (Column 3). By expressing these total excretions in per cent of the absorbed oral dose, the figures listed in the left half of Column 4 were obtained: 74 to 21 per cent of the absorbed oral dose was excreted. The good agreement between these figures and those obtained following the intravenous dose is evident. However, it should be

TABLE IV

METABOLISM OF ORAL AND INTRAVENOUS $Sr^{85}$
A 12 DAY STUDY

| CASE No. | ABSORPTION OF ORAL $Sr^{85}$ % OF DOSE | EXCRETION OF % DOSE $Sr^{85}$ | | | COMPARISON OF EXCRETION OF $Sr^{85}$ % OF ABSORBED DOSE AFTER | |
|---|---|---|---|---|---|---|
| | | URINE | STOOL ENDOGENOUS | TOTAL | $Sr^{85}$, ORAL | $Sr^{85}$, I.V. |
| 1 | 23.4 | 14.8 | 2.4 | 17.2 | 74 | 68 |
| 2 | 20.0 | 8.8 | 2.5 | 11.3 | 56 | 48 |
| 3 | 25.4 | 8.4 | 3.5 | 12.0 | 48 | 46 |
| 4 | 19.8 | 2.9 | 1.3 | 4.2 | 21 | 21 |

emphasized that the calculation of the endogenous fecal strontium following the oral tracer dose is based on the assumption that the pattern of excretion of strontium with the digestive juices is similar following the intravenous or oral administration of the tracer (see above).

*Comparison of calcium and strontium metabolism*

The metabolism of calcium and strontium was compared in two patients (2 and 6, Table I). Although the isotopes were not administered simultaneously, the experimental conditions of the studies were comparable. Figure 2 illustrates the study of one of these patients (Patient 2). The plasma level of $Ca^{45}$ was higher than that of $Sr^{85}$ throughout the study, whereas the urinary excretion of $Ca^{45}$ was lower than that of $Sr^{85}$. The bargraphs illustrate the balance of these two tracers in 12 days: considerably less strontium than calcium remained in the tissues principally

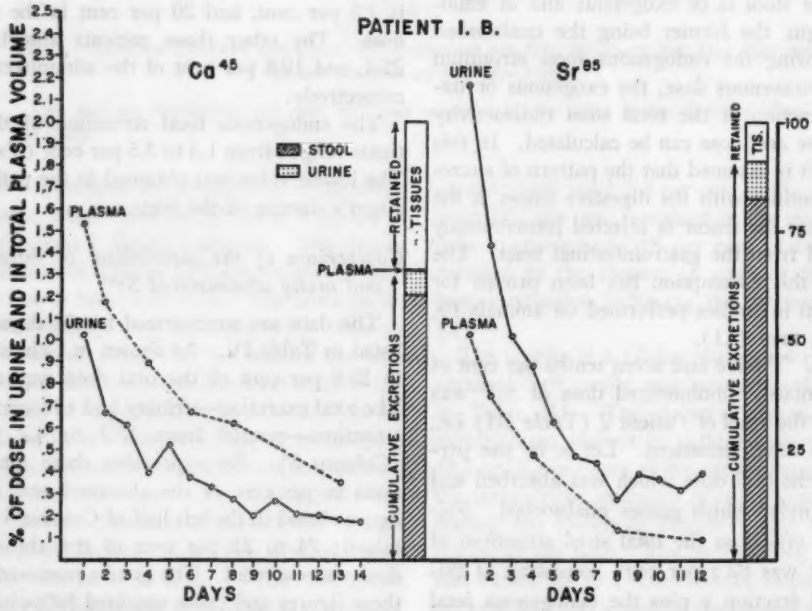


FIG. 2. METABOLISM OF $Ca^{45}$ AND $Sr^{85}$ FOLLOWING A SINGLE ORAL DOSE OF THE TRACERS

due to the higher fecal content and in part due to the higher urinary excretion of strontium.

Table V shows the balances of $Sr^{85}$ and $Ca^{45}$ of a 12-day study. A considerably greater amount of strontium than calcium is contained in the stool of both patients; the urinary strontium is almost twice as high as the urinary calcium excretion in I.B. (Patient 2) and slightly higher in B.L. (Patient 6). The latter patient retained both tracers better than the former. The ratio of calcium to strontium retention is approximately 4:1 and 2.5:1, respectively.

The absorption of strontium and calcium of the two patients is also compared in this Table. The fecal excretions were corrected for endogenous fecal strontium and calcium in patient I.B., and for endogenous fecal calcium in patient B.L. No correction could be made for endogenous fecal strontium in this latter patient since he has not received $Sr^{85}$ intravenously. However, the marked differences in absorption of the oral tracers can be readily seen: the absorption of both tracers is better in patient B.L., than in I.B., but only approximately half as much strontium as calcium is absorbed by each of the patients. Although less strontium than calcium is absorbed from the gastrointestinal tract, more strontium than calcium is excreted by the kidney as the figures of this Table indicate, the ratios being 4:1 and 2.5:1 for the two patients.

TABLE V

*Comparison of strontium and calcium excretions (cumulative) and of the absorption of the orally administered tracers, % dose—A 12-day study*

| | | I. B. | | B. L. | |
|---|---|---|---|---|---|
| | | $Sr^{85}$ | $Ca^{45}$ | $Sr^{85}$ | $Ca^{45}$ |
| Excretions | Stool | 82.51 | 60.15 | 70.60 | 39.45 |
| | Urine | 8.79 | 4.49 | 3.82 | 3.05 |
| | Total | 91.30 | 64.64 | 74.42 | 42.50 |
| Retention | | 8.70 | 35.36 | 25.58 | 57.50 |
| Absorption | | 20 | 43 | 30 | 67 |
| Per cent of *absorbed* dose excreted in urine | | 44 | 10 | 13 | 5 |

## DISCUSSION

The metabolism and skeletal uptake of strontium in man have received increasing attention in recent years for $Sr^{90}$, a $\beta$-emitting isotope with a 28-year half life is one of the major potential hazards of nuclear fission. Because of the radiation characteristics, $Sr^{90}$ could not be used for clinical investigation. Through the availability of $Sr^{85}$, a pure $\gamma$-emitter with a short half life, it became possible to investigate the metabolism and skeletal uptake of radiostrontium in man. Some of these results were recently published (1–4). The metabolism and skeletal uptake of radiostrontium were extensively studied in various species of *animals:* the isotope was fed or administered parenterally to young, adult and pregnant animals (12–15), the effect of various diets was investigated upon the metabolism of radioactive strontium (12, 14, 15) and the uptake in various parts of the skeleton was studied by radio-assay and autoradiographic techniques (12, 16, 17). Acute and chronic toxicity of radiostrontium were investigated (18–21). The metabolism and skeletal uptake of radioactive calcium and strontium were compared (16, 22–24) and the transport of these isotopes from maternal blood into the fetus was described (15, 22, 23).

Most of the metabolic data described in the experimental section agree well with those obtained in animals. For instance, the injected radiostrontium disappears rapidly from the blood stream (13, 15); the ingested tracer can be detected within a few minutes in the plasma, the radioactivity increases gradually, reaches a peak by 2 to 4 hours and declines slowly thereafter. Similar results were obtained with ingested radiocalcium (7, 8, 25). The major pathway of excretion of the *absorbed* radiostrontium is via the kidney, irrespective of the route of entry of the isotope. The maximum excretion takes place within 24 hours of the administration of the tracer by either route. The urinary excretion of radiostrontium varied from patient to patient. For instance, it was much higher in a patient who had carcinoma of the lung and was partially immobilized than in one with Paget's disease of the bone. The former excreted an average of 150 mg. calcium per 24 hours in the urine whereas the latter excreted only 30 mg. calcium per day. Similar observations on the correlation between the magnitude of urinary calcium and radiostrontium excretions were made on a large number of pa-

tients in our laboratory. These data indicate that the urinary strontium excretion reflects well the metabolic state of the skeleton. The rate of fecal losses of strontium via the digestive juice varies also but less than the urinary excretion so that the ratio of urinary to fecal loss was as high as 6:1 and as low as 2:1; this was mainly due to the differences in the rate of urinary strontium excretion. Under exceptional conditions such as in sprue syndrome or in patients with extensive osteoblastic metastases secondary to cancer of the prostate the urinary strontium excretion may be so low as to be exceeded by the endogenous fecal strontium.

Following the oral administration of radiostrontium the predominance of urinary over fecal strontium excretion is masked by the large fraction of *unabsorbed* radiostrontium of the stool. This predominance becomes apparent only if this unabsorbed fraction is subtracted from the total stool radioactivity and the endogenous fecal strontium is calculated. Approximately 80 per cent of the oral tracer passes unabsorbed. Comparison of the metabolism of ingested and parenterally administered radiostrontium revealed good agreement of the data.

Until recently only scanty information was available in the literature on the metabolism of stable strontium. Harrison, Raymond, and Tretheway reported on the study of five volunteers who were given 20 to 250 mg. $Sr^{++}$ by the intravenous or oral route (26). These authors emphasize the differences between experiments performed with carrier amounts of strontium and tracer studies and feel that the latter are only of value as a means of assessing radiotoxicity. However, their data are in good agreement with those reported in this communication employing the radioactive tracer except that our studies have proven that significant amounts of strontium are carried with the digestive juices into the gastrointestinal tract. Also, the higher rate of urinary strontium excretion in the study of the English investigators may in part be due to the unphysiologic amounts of carrier strontium administered since enhancement of urinary strontium excretion was recently reported to occur following the administration of unphysiologic amounts of carrier calcium (1, 2). Furthermore, the high calcium intake and the high calcium clearance may also have contributed to the high urinary strontium excretion of their volunteers. McCance and Widdowson suggested that strontium behaves physiologically like calcium (27). Shorr and Carter studied metabolic balances in patients with osteoporosis receiving varying amounts of calcium and strontium supplements orally (28). They found that strontium is a valuable adjuvant in the remineralization of the skeleton of these patients.

However, no data are available on the direct comparison of the metabolism of radiostrontium and radiocalcium in man. Studies on the two patients reported here who received both tracers orally disclosed that major differences do exist. Radiocalcium was found to be more efficiently absorbed than radiostrontium and, in spite of the poor absorption of the latter, its renal clearance was significantly higher than that of calcium so that the remaining body load of strontium was only one-fourth to one-half of that of calcium. The data obtained in humans are in agreement with those obtained in animals by Comar, Whitney, and Lengeman (23) and by Bauer, Carlsson, and Lindquist (24). The latter investigators report that the body load of $Sr^{90}$ in rats is significantly lower than of $Ca^{45}$ due to the higher renal clearance, and higher gastrointestinal excretion of strontium. However, they claim that the skeleton *per se* cannot differentiate between calcium and strontium and the differences in skeletal uptake are secondary to the higher renal clearance. Harrison, Raymond and Tretheway believe that the absorption of calcium from the gastrointestinal tract of man is at least twice as great as that of strontium (26).

The studies carried out with radioactive or stable strontium indicate that strontium cannot be used as an accurate indicator for calcium metabolism. Its possible use as an investigative tool in physiologic and pathologic states of the skeleton of man is currently under investigation. These data, analyzing plasma disappearance rates, renal clearance and digestive juice losses of radiostrontium and of radiocalcium will be reported in a subsequent communication. Also, balances of intake and excretion do not provide information on the localization of the retained element. Therefore, human tissues obtained at autopsy have been radioassayed for calcium and strontium. Some of these data have been recently presented (29).

SUMMARY

1. The metabolism of an orally administered tracer of radiostrontium was studied in 6 patients and compared with that of an intravenous tracer in four. The metabolism of an orally administered tracer dose of radiocalcium and of radiostrontium was studied in two patients.

2. The main pathway of excretion of $Sr^{85}$ is via the kidney irrespective of the route of administration of the isotope and the urinary $Sr^{85}$ excretion differs in various subjects depending on the state of the metabolism of the skeleton.

3. Another pathway of excretion of strontium is the intestinal tract: approximately 10 per cent of the absorbed strontium is eliminated via this route, *i.e.*, endogenous fecal strontium.

4. The absorption of ingested radiostrontium was determined by subtracting the endogenous fecal strontium from the stool radioactivity. Strontium is poorly absorbed from the gastrointestinal tract, an average of 80 per cent of the dose passes unabsorbed.

5. The metabolism of the absorbed fraction of the oral dose of $Sr^{85}$ agreed well with that of the intravenous dose. The body load of strontium is approximately four to six times higher after the intravenous than after the oral dose.

6. Comparison of the metabolism of oral $Sr^{85}$ and $Ca^{45}$ revealed that calcium is better absorbed than strontium while the latter is preferentially excreted. For these reasons—poorer absorption and preferential excretion—significantly less strontium than calcium remains in the human body.

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# INCREASED ALDOSTERONE SECRETION IN DOGS WITH RIGHT-SIDED CONGESTIVE HEART FAILURE AND IN DOGS WITH THORACIC INFERIOR VENA CAVA CONSTRICTION

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Studies (1–5) of patients with congestive heart failure, decompensated hepatic cirrhosis and nephrosis have provided evidence of increased amounts of aldosterone or aldosterone-like substances in urine during edema or ascites formation. More recently, it has been demonstrated (6) that increased urinary excretion of aldosterone occurs in association with sodium retention in dogs with right heart failure and in dogs with ascites secondary to constriction of the thoracic inferior vena cava. The data in both man and dog imply that the blood level of aldosterone is elevated.

Numerous attempts have been made to elucidate the mechanism regulating aldosterone secretion in clinical states associated with the formation of edema, but no data have been reported to show that increased circulating aldosterone results from increased secretion rather than from a decreased rate of inactivation. The purpose of this study was to determine the relative rates of secretion of aldosterone in 1) dogs with cardiac failure produced by stenosis of the pulmonary artery, 2) dogs with thoracic inferior vena cava constriction and ascites, and 3) normal dogs.

## METHODS

Right heart failure was produced in four dogs by controlled progressive constriction of the main pulmonary artery by a method described previously (7). In three other animals, ascites formed following constriction of the thoracic inferior vena cava. Six normal dogs provided the control material. All animals were female mongrel dogs weighing 15 to 23 kg. The dogs were fed a synthetic diet containing 60 mEq. of sodium and 18 mEq. of potassium per day with two exceptions; one dog with heart failure and one normal dog received 40 mEq. per day of sodium and 40 mEq. per day of potassium. At the time adrenal vein blood was obtained, all dogs with heart failure or caval constriction had a high venous pressure; ascites was present and urinary sodium excretion was less than 10 mEq. per day.

Adrenal vein blood was obtained under two sets of circumstances. First, blood was collected with the animals under sodium pentobarbital anesthesia; no infusion was given during the procedure. In the second group of animals, adrenal vein blood was collected with the dogs awake. In this group, a local anesthetic (Xylocaine) was used for the skin and muscle of the abdominal wall but none was injected near the adrenal gland; morphine sulfate (5 to 10 mgm.) was given subcutaneously to all but one dog in the second group. This series of dogs received a blood transfusion at approximately the same rate that adrenal venous blood was obtained. Peripheral blood was collected from two of the dogs of the second group. The peripheral blood was obtained from the dog with cardiac failure immediately after collection of adrenal vein blood and from the dog with thoracic caval constriction on the day before collection of adrenal vein blood.

Adrenal venous blood (300 cc.) was collected by cannulating the right adrenolumbar vein with a polyethylene catheter located peripheral to the point of venous inflow from the right adrenal gland. All tributaries to the adrenolumbar vein in this region other than the adrenal veins were ligated and the adrenolumbar vein was occluded at its junction with the inferior vena cava. By this procedure, flow of venous blood from the right adrenal gland was diverted from its normal pathway into the polyethylene catheter. Heparin (40 to 50 mgm.) was injected intravenously into each animal before collection of blood was begun. Flow was measured by collecting blood in a graduated cylinder. The cylinder was immersed in an ice bath and each 25 cc. collection was transferred immediately to a flask of acetone surrounded by ice.

The procedure for isolation of aldosterone from adrenal venous and peripheral blood is shown in Figure 1. An aqueous acetone extract of the blood was concentrated by evaporation in an atmosphere of nitrogen and partitioned twice between petroleum ether and aqueous 70 per cent ethanol. The aqueous ethanol extract was similarly concentrated and then extracted with methylene chloride. This methylene chloride extract was concentrated and

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subjected to column partition chromatography, with ethylene glycol as the stationary phase and mixtures of methylene chloride and cyclohexane as the mobile phase. No attempt was made to remove compound E from the aldosterone fraction since compound E does not interfere with the assay for aldosterone (8) unless the amount present far exceeds that found in adrenal vein blood from dogs (9). Also, the combination of compound E and aldosterone produces less Na retention by assay than does aldosterone alone (8).

The various chromatographic fractions of adrenal vein and peripheral blood were assayed in bilaterally adrenalectomized female dogs according to the assay procedure of Liddle, Cornfield, Casper, and Bartter (8) except that the crossover design was not employed. The adrenalectomized dogs were maintained on a diet containing 200 mEq. per day of sodium and 18 mEq. per day of potassium; 5 mgm. per day of cortisone acetate were given intramuscularly 18 hours before the assay. Sodium-retaining and potassium-excreting activity obtained by assay was expressed in terms of aldosterone or aldosterone equivalents which were determined from a dose-response curve obtained previously (6).

## RESULTS

A comparison of the concentration of aldosterone in peripheral and adrenal vein blood is presented in Figure 2. Adrenal vein blood from the dog with cardiac failure contained 12.3 μg. in 300 cc. while blood from the dog with thoracic caval constriction showed 5.0 μg. in 300 cc. No aldosterone was detected in the same quantity of peripheral blood from either dog. The data demonstrate that the aldosterone present in adrenal venous effluents was secreted aldosterone rather than circulating hormone.

Studies of aldosterone production demonstrated a higher rate of secretion in dogs with thoracic caval constriction or heart failure than in normal dogs (Table I). The initial observations were made using sodium pentobarbital anesthesia to obtain adrenal vein blood. In normal animals, aldosterone was not detected in 300 cc. of adrenal vein blood; the assay values were less than 4.0 μg. of aldosterone equivalents and were of the same order of magnitude for all chromatographic fractions. In contrast, aldosterone was present in concentrations of 9.0 and 15.3 μg. per 300 cc. of adrenal vein blood from the dog with thoracic caval constriction and from the dog with heart failure. The rates of aldosterone secretion were 6.0 and 4.9 μg. per hr., respectively, for these two animals.

Observations on aldosterone secretion using local anesthesia also showed a higher rate of secretion in dogs with caval constriction or heart failure than in normal dogs (Table I). In addition, the rates of aldosterone production were higher during local anesthesia in both normal dogs and dogs with heart failure or caval constriction than

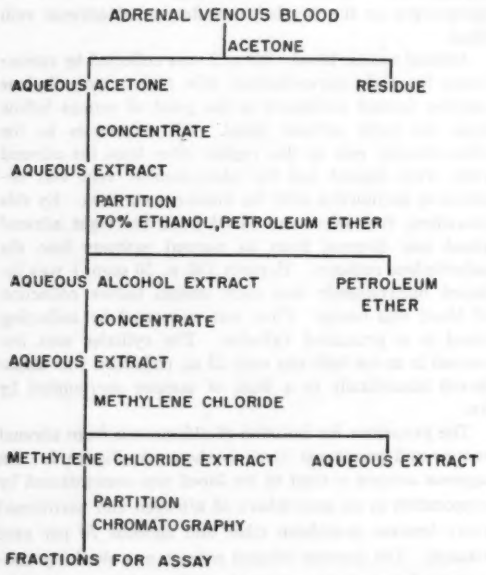


FIG. 1. METHOD OF EXTRACTION OF ADRENAL VEIN AND PERIPHERAL BLOOD

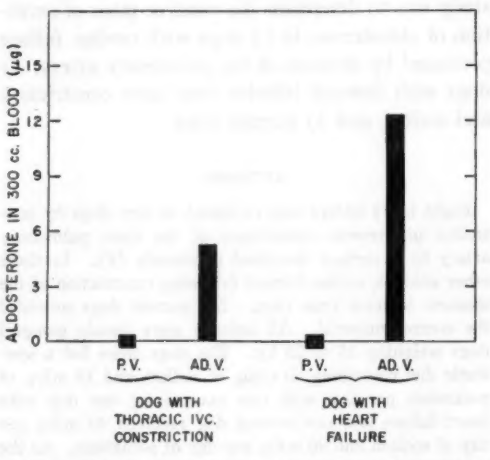


FIG. 2. COMPARISON OF THE ALDOSTERONE CONTENT OF PERIPHERAL VEIN (P.V.) AND ADRENAL VEIN (AD.V.) BLOOD

TABLE I

*Results from assay of chromatographic fractions of adrenal vein blood and measurements of adrenal blood flow, aldosterone secretion and venous pressure*

| Dogs | Venous pressure *mm. water* | Aldosterone equivalents in μg./300 cc. adrenal vein blood: Fraction II Compound DOC | III A | IV B + S | V† | VI VII Aldosterone +E | VIII F | Adrenal blood flow *cc./min.* | Aldosterone secretion *μg./hr.* |
|---|---|---|---|---|---|---|---|---|---|
| *During sodium pentobarbital anesthesia* | | | | | | | | | |
| Normal | | * | | * | * | * | * | 2.54 | |
| Normal | | * | * | * | * | * | * | 6.25 | |
| Normal | | —— | —— | * | —— | * | * | 4.17 | |
| Thoracic caval constriction | 230‡ | * | * | * | * | 9.0 | * | 3.33 | 6.0 |
| Heart failure | 150 | —— | —— | * | —— | 15.3 | * | 1.58 | 4.9 |
| *During local anesthesia* | | | | | | | | | |
| Normal | | * | | —— | * —— | * | * | 2.76 | |
| Normal | | * | | —— | 5.0 —— | 8.4 | * | 2.88 | 4.8 |
| Normal | | 4.8 | | —— | 4.2 —— | 4.4 | * | 3.55 | 3.2 |
| Thoracic caval constriction | 210 | * | | —— | * —— | 5.0 | * | 6.66 | 6.8 |
| Thoracic caval constriction | 150 | * | | —— | * —— | 37.3 | * | 2.19 | 16.4 |
| Heart failure | 240 | —— | —— | * | —— | 21.0 | * | 5.83 | 24.5 |
| Heart failure | 240 | —— | —— | 4.7 | —— | 35.0 | * | 2.88 | 20.2 |
| Heart failure | 270 | * | | —— | * —— | 12.3 | * | 1.92 | 4.7 |

* Indicates that biological activity by assay was less than 4.0 μg. of aldosterone equivalents per 300 cc. of adrenal vein blood; 4.0 μg. is the smallest amount of activity detectable by this assay procedure.

† Fraction V was obtained by using the same mixture of methylene chloride and cyclohexane as for Fraction IV.

‡ Values for venous pressure were obtained at the beginning of the collection of adrenal vein blood. The normal range of venous pressure is 35 to 70 mm. water (7).

during sodium pentobarbital anesthesia. Aldosterone was found in adrenal vein blood collected under local anesthesia from two of three normal dogs and the rates of secretion were 3.2 and 4.8 μg. per hr. No aldosterone was detected in adrenal vein blood from the other normal dog. These findings in normal dogs are in contrast with the high rates of aldosterone secretion of 6.8 and 16.4 μg. per hr. for the dogs with caval constriction and of 4.7, 20.2 and 24.5 μg. per hr. for the dogs with heart failure. Statistical comparison of the rate of aldosterone secretion in 6 normal dogs with that in the 7 dogs with either caval constriction or heart failure revealed a significant difference ($T = 3.0$; $P < 0.02$).

The results from assay of fractions of adrenal vein blood other than the aldosterone fraction showed no consistent evidence of activity causing sodium retention or increased potassium excretion for any fraction. It should be pointed out also that three of the four non-aldosterone fractions which showed activity were from adrenal vein blood from normal dogs. Consequently, the data demonstrate that increased biological activity of this type in adrenal vein blood from dogs with heart failure and from dogs with caval constriction was limited to the aldosterone fraction.

## DISCUSSION

As early as 1941, Raab (10) reported that an increased blood level of adrenocortical hormones occurs in patients with congestive heart failure. During the next few years very little consideration was given the possible role of the adrenal cortex in the physiology of heart failure. Instead, with the availability of methods for measuring renal hemodynamic function in the intact unanesthetized animal, interest developed in the possible importance of the effect of a reduced rate of glomerular filtration on sodium excretion. In 1950, however, Deming and Luetscher (1) reported the presence of increased sodium-retaining activity in urine from patients with cardiac failure. There followed several studies (11–15) which provided very strong indirect evidence from measurements of electrolytes in sweat, saliva, urine, and

feces that in patients with cardiac failure or decompensated hepatic cirrhosis adrenocortical hormones promoting sodium retention are present in increased amounts. Also, there were additional reports that increased sodium-retaining activity is present in urine from patients with cardiac failure (3), cirrhosis (2) and nephrosis (4). Since the isolation and crystallization of aldosterone in 1952, it has been shown that the excretion of aldosterone in urine from patients (16) and from dogs (6) accumulating edema or ascites is increased. Furthermore, a rough inverse correlation of aldosterone output with sodium excretion in urine was described by Luetscher and Johnson (5). The association of increased aldosterone excretion in urine with renal sodium retention was also demonstrated by studies of aldosterone and sodium excretion before and after bilateral adrenalectomy in dogs with caval constriction and ascites (6). During sodium retention in dogs with caval constriction and intact adrenals, aldosterone excretion was elevated above normal, but following bilateral adrenalectomy and discontinuation of hormone therapy aldosterone disappeared from urine and a natriuresis resulted. More quantitative data on the relation of an increased blood level of a sodium-retaining steroid to sodium excretion during ascites formation has been presented previously for adrenalectomized dogs with cardiac failure (17) and for adrenalectomized dogs with caval constriction (15). In both animal preparations it was found that the degree of sodium retention was directly related to the amount of desoxycorticosterone acetate (DCA) administered. Consequently, available evidence indicates a causal relationship between increased circulating aldosterone and sodium retention during cardiac failure and during ascites formation.

It should be pointed out also that increased sodium-retaining hormone alone is insufficient to produce chronic sodium retention in the dog and in some patients with primary aldosteronism (18). Large doses of DCA failed to produce more than transient retention of sodium in normal (19) and in adrenalectomized dogs (20). In the presence of an elevated venous pressure, however, as in the adrenalectomized dog with constriction of the thoracic inferior vena cava (15) or in the adrenalectomized dog with cardiac failure (17), large doses of DCA produced almost complete sodium retention and massive ascites.

The present study demonstrates that aldosterone is secreted in dogs with cardiac failure and in dogs with caval constriction at a rate considerably higher than in normal dogs. Failure to detect aldosterone in peripheral blood although large amounts were present in adrenal vein blood shows that the increased amounts of aldosterone in the adrenal venous effluents resulted from increased secretion and were not representative of the blood level of circulating aldosterone. The observations made during local anesthesia and during blood transfusion provide an estimate of the rate of aldosterone secretion for comparison with urinary aldosterone output. For the data to be meaningful in terms of the average rate of aldosterone secretion during the day, it was necessary to collect blood with the dogs awake; the present data suggest that sodium pentobarbital depressed the rate of aldosterone secretion. Also, Farrell, Rosnagle, and Rauschkolb (21) demonstrated that bleeding stimulates the adrenal cortex to secrete aldosterone. In the present study, a transfusion was given to replace the blood removed and, consequently, to prevent the effect of bleeding *per se* on aldosterone secretion. If the average value for the rate of aldosterone secretion per hour is used to provide a rough estimate of daily secretion, the rates of aldosterone secretion were 560 $\mu$g. per day for the dogs with caval constriction and 780 $\mu$g. per day for the dogs with heart failure. The rate of excretion of aldosterone in urine from dogs with heart failure and from dogs with constriction of the thoracic inferior vena cava varied from 2 to 6 $\mu$g. per day (6). Similar calculations from the data on normal dogs yield a value of 130 $\mu$g. per day for the rate of aldosterone secretion. No aldosterone was detected in 4 days of normal dog urine but chromatographic fractionation and assay of a 26-day pool of normal dog urine showed that aldosterone was present (6). It appears, therefore, that only 1 per cent or less of secreted aldosterone is excreted in urine from the dog. These findings call attention to the limitation of studies of urinary excretion for evaluation of the factors which regulate the rate of aldosterone secretion.

The sequence of events between the primary myocardial defect in heart failure or the primary

hepatic defect in cirrhosis of the liver [2] and the mechanism which stimulates the adrenal cortex to secrete aldosterone has not been clearly delineated. In the dog with caval constriction an elevated venous pressure below the constricting ligature is usually the only detectable alteration in cardiovascular function (22). This finding suggests that elevated venous pressure initiates the sequence of events leading to increased secretion of aldosterone and the subsequent renal retention of sodium. Previous work (22, 23) indicated that the influence of venous pressure on the adrenal cortex is indirect; hepatic venous congestion, which is essential to the formation of ascites with a high protein content, appears to be the important consequence of venous hypertension. Whereas sufficient constriction of the thoracic inferior vena cava above the hepatic venous outflow produces ascites consistently (24), constriction of the inferior vena cava above the renal (24, 25) or adrenal (23) veins but below the diaphragm or constriction of the hepatic portal vein (23–24) fails to result in chronic sodium retention. The importance of the liver may be related to the permeability of its vascular and lymphatic beds which are areas where fluid and electrolytes escape readily from the blood stream. It is uncertain whether the initial ascites forms before or after a positive sodium balance is established by the kidney but observations have shown that the first alternative is possible. In both dogs with caval constriction and dogs with pulmonic stenosis, the initial formation of ascites was observed in the absence of sodium intake; ascites formed during the first 4 days after caval constriction or pulmonic stenosis while food was withheld (26). The extravasated fluid and electrolytes and resultant ascites formation were associated with increased urinary excretion of aldosterone. The findings from this series of studies are consistent with the hypothesis proposed by Peters several years ago (27) that an elevated venous pressure, extravasation of fluid and electrolytes, adrenocortical stimulation with the consequent increased secretion of a sodium-retaining hormone, and sodium retention occur in this order.

[2] The dog with thoracic inferior vena cava constriction and ascites resembles closely the patient with decompensated hepatic cirrhosis in regard to electrolyte metabolism.

## SUMMARY AND CONCLUSIONS

Adrenal vein blood was collected from six normal dogs, three dogs with thoracic inferior vena cava constriction and four dogs with right-sided congestive heart failure. Chromatographic fractionation of the blood and assay of the various fractions showed activity causing sodium retention and increased potassium excretion to be present consistently in only one fraction, the aldosterone fraction. Studies of aldosterone in peripheral and adrenal vein blood from the same animals with either heart failure or thoracic caval constriction demonstrated aldosterone in adrenal venous blood only; it may be concluded, therefore, that aldosterone present in adrenal vein blood represents secreted aldosterone. The rates of aldosterone secretion were significantly greater for dogs with thoracic caval constriction or dogs with heart failure than for normal dogs. From data obtained using local anesthesia, the average rate of aldosterone secretion for normal dogs was 2.7 μg. per hr., for dogs with caval constriction, 11.6 μg. per hr., and for dogs with heart failure, 16.5 μg. per hr.

## ACKNOWLEDGMENTS

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# THE DISTRIBUTION OF $Cr^{51}$ LABELED *E. COLI* ENDOTOXIN IN THE GENERALIZED SHWARTZMAN REACTION [1]

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The generalized Shwartzman reaction (1) occurs in immature (2) rabbits following the second of two sublethal intravenous injections of gram-negative bacterial endotoxin, spaced 18 to 24 hours apart, and consists of widespread hemorrhage and necrosis. The reaction also occurs after a single injection of endotoxin in animals treated with intravenous thorotrast, trypan blue (3), colloidal iron, or colloidal carbon (4). Because these substances were thought to "blockade" the reticuloendothelial (R.E.) system, it was suggested that an analogous impairment of R.E. function may also be produced by the first or "preparing" injection of endotoxin, resulting in slower removal and detoxification of the second dose and directly or indirectly in the characteristic lesions in the kidney, heart, lungs, and liver.

In the experiments to be reported, the distribution of radioactive $Cr^{51}$ labeled endotoxin in normal rabbits was compared with that in rabbits which had received a preparing injection of unlabeled endotoxin 24 hours earlier. The results indicate that, in the rabbit, the "prepared" state is characterized by impaired removal of a second injection of endotoxin by the liver and increased localization by the lung.

## METHODS AND PROCEDURE

*Animals.* Young albino rabbits of a hybrid stock weighing between 0.77 and 1.3 kilo., used for all experiments, were housed in air-conditioned, humidity-controlled animal quarters and fed water and Purina rabbit pellets.

*Endotoxin.* *Escherichia coli* endotoxin was prepared and labeled with radioactive chromic chloride ($Cr^{51}Cl_3$) according to the methods previously reported (5). The close quantitative relationship shown to exist between the toxicity and radioactivity of endotoxin so labeled provides the basis for using radioactivity as a measure of the distribution of endotoxin. In preliminary experiments it was found that the lot of endotoxin employed in these experiments produced the generalized Shwartzman reaction with high frequency in immature rabbits.

*Experimental.* Each rabbit in one experimental group received an intravenous injection of 0.25 mgm. non-labeled *E. coli* endotoxin in the marginal vein of the ear (the "preparing" injection); 24 hours later each prepared animal received a second intravenous injection of 0.56 mgm. *E. coli* endotoxin labeled with $Cr^{51}Cl_3$. In a second group of animals, each received an intravenous injection of 0.56 mgm. labeled endotoxin, but no preparing injection of non-labeled endotoxin.

At varying intervals after the injection of radioactive endotoxin, samples of blood were removed by cardiac puncture, heparinized, and 5.0 ml. plasma saved for determination of radioactivity. The animals were immediately sacrificed and the weights of each animal and its individual organs were recorded. The lung, heart, spleen, kidney, and weighed aliquots of liver were rinsed with tap water to remove superficial blood and placed in 10 per cent formol-saline until radioactivity was measured. After counting, sections of the organs, stained with hematoxylin and eosin were examined microscopically. When the volume of an organ made it necessary to digest with concentrated nitric acid before counting, a weighed aliquot was first removed for histologic section. The radioactivity of the tissues and of injected labeled endotoxin was determined by counting in a scintillation counter for ten minutes as previously described (5). Corrections were made for decay. On the basis of previous data obtained in rabbits given endotoxin, total circulating plasma radioactivity was calculated by assuming an average hematocrit of 40 per cent and an average blood volume of 5 per cent of body weight (6).

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TABLE I

*Comparison of uptake of radioactive $Cr^{51}$ labeled endotoxin in normal rabbits and those which received non-labeled endotoxin twenty-four hours earlier*

| Rabbit number | Interval between injection and sacrifice | % Injected radioactivity* | | | | | | Concentration index† | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Plasma 5 ml. | Spleen | Liver | Lung | Heart | Kidney | Spleen | Liver | Lung | Heart | Kidney |
| Group I—0.25 mgm. *E. coli* endotoxin, I. V. 24 hours prior to 0.56 mgm. $Cr^{51}$ labeled endotoxin I. V. | | | | | | | | | | | | |
| 16 | 35′ | 3.8 | 1.2 | 41.7 | 3.0 | 0.2 | | 17.1 | 7.1 | 4.3 | | |
| ‡17 | 38′ | 5.8 | 2.7 | 21.9 | 4.0 | 0.5 | 3.6 | 13.0 | 6.2 | 3.5 | 2.1 | 2.3 |
| ‡18 | 75′ | 3.5 | 4.5 | 28.1 | | 0.3 | 1.8 | 20.3 | 6.1 | | 1.7 | 1.0 |
| ‡20 | 77′ | 3.9 | 1.3 | 21.7 | | 0.2 | 0.6 | 16.4 | 5.6 | | 1.1 | 0.5 |
| 21 | 123′ | 2.4 | 1.3 | 49.7 | 1.7 | 0.2 | 0.7 | 21.8 | 11.2 | 2.8 | 1.2 | 0.8 |
| ‡22 | 125′ | 2.7 | 4.7 | 25.7 | 4.4 | 0.4 | 2.0 | 27.4 | 10.3 | 5.4 | 1.7 | 1.5 |
| ‡23 | 180′ | 3.5 | 1.6 | 31.9 | 2.3 | 0.1 | 1.9 | 19.5 | 8.5 | 3.1 | 0.7 | 1.9 |
| 24 | 185′ | 2.7 | 6.5 | 24.4 | 6.1 | 0.3 | 0.3 | 49.9 | 5.8 | 7.2 | 1.2 | 0.3 |
| ‡25 | 255′ | 5.1 | 1.3 | 30.6 | | | 0.3 | 14.6 | 7.3 | | | 3.0 |
| 26 | 257′ | 2.3 | 2.0 | 29.4 | 2.5 | 0.3 | 0.4 | 22.9 | 6.75 | 3.2 | 1.5 | 0.4 |
| 27 | 363′ | 1.4 | 13.7 | 29.3 | 3.2 | 0.2 | 0.5 | 57.1 | 7.0 | 3.4 | | |
| ‡28 | 364′ | 2.6 | 2.9 | 40.3 | 6.0 | 0.1 | 1.3 | 29.0 | 9.3 | 7.0 | 0.4 | 1.1 |
| §30 | 11° | 0.7 | 1.9 | | 4.0 | 0.1 | 3.9 | 15.8 | | 4.1 | 0.4 | 3.2 |
| ‡31 | 11° | 2.6 | 1.3 | | 1.4 | 0.4 | 0.4 | 11.0 | | 1.7 | 1.9 | 0.4 |
| ‡32 | 18° | 1.2 | 2.4 | 34.4 | 2.4 | 0.1 | 0.9 | 11.9 | 8.5 | 3.4 | 0.8 | 1.1 |
| §33 | 24° | 0.5 | 3.6 | | 2.1 | 0.3 | 2.1 | 23.8 | | 2.3 | 1.4 | 2.0 |
| ‡34 | 24° | 0.9 | 5.6 | 28.9 | | 0.1 | 0.4 | 5.1 | 8.6 | | | |
| §35 | 15° | | 6.9 | 27.9 | 2.8 | 0.4 | 7.0 | 36.5 | 5.3 | 2.5 | 1.8 | 4.8 |
| Group II—Single injection I. V. 0.56 mgm. $Cr^{51}$ labeled endotoxin | | | | | | | | | | | | |
| 36 | 41′ | 3.1 | 2.1 | 46.9 | | 0.3 | 1.7 | 38.5 | 8.3 | | 1.7 | 2.2 |
| ‡37 | 43′ | 4.4 | 3.6 | 42.6 | 0.2 | 0.3 | 1.4 | 36.2 | 15.9 | 0.4 | 1.7 | 1.9 |
| 38 | 79′ | 5.3 | | 46.9 | 0.6 | 0.4 | 0.6 | | 7.4 | 1.3 | 1.9 | 0.7 |
| 39 | 81′ | 5.0 | 3.6 | 57.5 | 0.8 | 0.4 | 1.2 | 29.8 | 10.8 | 1.1 | 2.2 | 1.5 |
| 40 | 128′ | 1.4 | 2.6 | 68.1 | 1.9 | 0.2 | 0.4 | 28.3 | 14.7 | 3.3 | 0.9 | 0.4 |
| ‡41 | 130′ | 4.0 | 1.9 | 57.5 | 0.5 | 0.4 | 0.8 | 28.4 | 10.4 | 0.8 | 1.4 | 0.8 |
| 42 | 190′ | 2.8 | 2.5 | 53.1 | 3.2 | 0.3 | 1.7 | 13.9 | 13.4 | 5.0 | 1.2 | 1.0 |
| ‡43 | 192′ | 4.0 | 1.1 | 45.6 | 2.1 | 0.2 | 1.5 | 15.4 | 13.4 | 3.0 | 0.9 | 1.7 |
| 44 | 262′ | | 0.9 | 31.9 | 3.2 | 0.2 | 0.3 | 5.5 | 10.2 | 4.9 | 1.3 | 0.4 |
| 45 | 264′ | 3.1 | 2.4 | 58.6 | 1.9 | 0.2 | 1.0 | 26.1 | 17.9 | 2.4 | 1.1 | 1.2 |
| 46 | 367′ | | 1.6 | 57.5 | | 0.1 | 0.8 | 31.5 | 11.7 | | 0.4 | 0.8 |
| ‡47 | 369′ | 3.0 | 2.0 | 47.4 | 1.9 | 0.3 | 0.3 | 28.3 | 8.9 | 2.6 | | |
| §48 | 11° | 2.4 | 2.3 | | 0.9 | 0.5 | 1.9 | 38.9 | | 1.6 | 1.9 | 1.8 |
| 49 | 11° | 2.5 | 2.0 | 50.4 | 1.1 | 0.2 | 0.5 | 25.8 | 12.2 | 1.8 | 0.7 | 0.4 |
| 50 | 18° | 1.1 | 2.7 | 54.4 | 0.8 | 0.3 | 0.1 | 37.5 | 13.0 | 1.2 | | |
| 51 | 18° | 1.7 | 1.4 | 41.1 | 1.9 | 0.2 | 1.4 | 18.2 | 10.6 | 0.6 | | 1.7 |
| 52 | 24° | 0.9 | 1.9 | 67.7 | | 0.2 | 0.2 | 25.8 | 19.2 | | | |
| 53 | 24° | 1.2 | 1.6 | 45.5 | 0.7 | 0.4 | 1.1 | 27.2 | 11.9 | 0.9 | 2.0 | 1.7 |

* $\frac{\text{c.p.s. in total organ}}{\text{c.p.s. injected}} \times 100 = \%$ Injected radioactivity in given organ.

† $\frac{\text{Per cent of injected radioactivity in given organ}}{\text{Per cent of total body weight in organ}} =$ Concentration index.

‡ Fibrinoid deposits in glomeruli but no necrosis detectable microscopically.

§ Bilateral renal cortical necrosis present grossly and microscopically.

## RESULTS

The results of these studies are given in detail in Table I and presented graphically in Figures 1, 2, 3, and 4.

The distribution of radioactive endotoxin in the normal rabbits resembles that reported previously (6). Figure 1 shows that clearance of radioactivity from the plasma of the prepared animals did not differ significantly from normals. On the other hand, uptake of radioactivity by the liver (Figure 2) was strikingly reduced in the prepared animal. Hepatic uptake accounted for as much as 60 per cent of injected radioactivity in the normal rabbit, whereas uptake in the prepared rabbit was usually less than 40 per cent. Figure 4 shows that the lung of the prepared rabbit accumulates a higher percentage of the injected radioactivity than the normals.

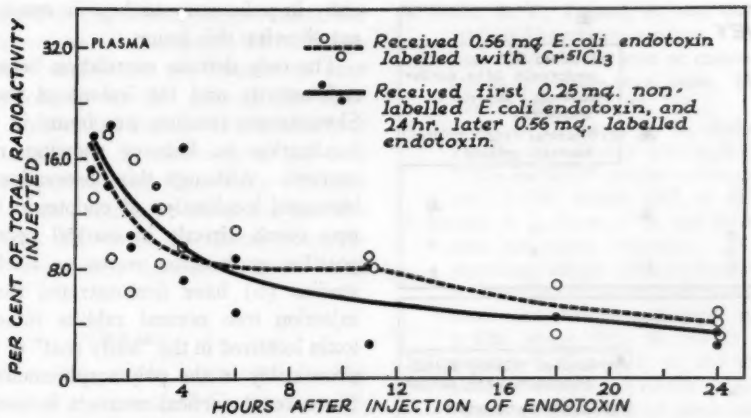



FIG. 1. PER CENT OF INJECTED RADIOACTIVITY PRESENT IN PLASMA AT VARIOUS TIMES AFTER INTRAVENOUS INJECTION OF $Cr^{51}$ LABELED ENDOTOXIN, IN NORMAL RABBITS AND ANIMALS WHICH HAD RECEIVED A PREPARING INJECTION OF 0.25 MGM. UNLABELED ENDOTOXIN 24 HOURS EARLIER

Each point represents the value for a single animal.

Uptake of radioactive endotoxin by the kidneys in the two groups did not differ significantly except in the rabbits which developed bilateral renal cortical necrosis (Figure 3). The kidneys of four such animals contained an average of 3.72 per cent of radioactivity injected, whereas the kidneys of those living more than 6 hours and not showing renal cortical necrosis contained an average of only 0.62 per cent. This apparent increase in uptake of radioactivity is not explained simply by the increased size of the involved kidneys, because the average concentration index was also increased in that group. Excluding those developing necrosis, glomerular fibrinoid deposits were found in the kidneys of 5 single-dose rabbits, but in 13 prepared animals. In contrast, no significant differences in localization of radioactivity by these two groups were observed.

Bilateral renal cortical necrosis occurred with the expected incidence (3 of 6 living more than 6 hours) in the prepared group, but one animal also showed this lesion after one injection of endotoxin. Presumably, the fibrinoid deposits occluding the glomerular lumen as a result of a single injection of endotoxin were sufficiently extensive to result in cortical necrosis (7).

Radioactivity in the spleen in the two groups varied considerably, but the mean values were slightly higher for the prepared group than for the controls. (Mean uptake in prepared group equalled 3.63 per cent, in controls, 2.11 per cent.)

Not all of the injected radioactive endotoxin was accounted for in the organs examined and it seems possible that localization may have occurred in the bone or other points in the carcass not examined. On the basis of previous data (6), intestinal or renal excretion probably would not account for a significant amount of endotoxin.

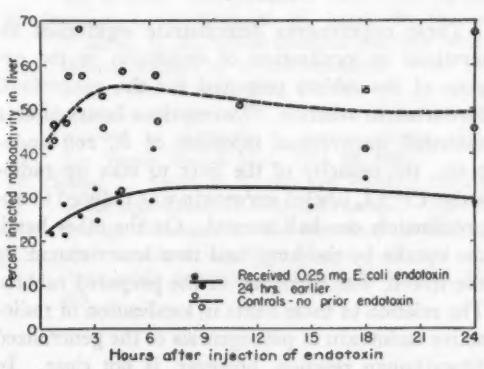
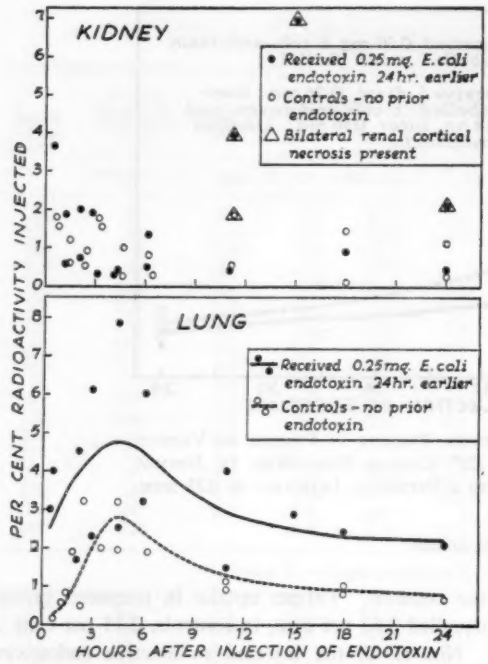



FIG. 2. PER CENT OF INJECTED RADIOACTIVITY LOCALIZED IN THE LIVER AT VARIOUS TIMES AFTER AN INTRAVENOUS INJECTION OF $Cr^{51}$ LABELED ENDOTOXIN IN NORMAL AND PREPARED RABBITS

Each point represents the value for a single animal.


KIDNEY
• Received 0.25 mg. E. coli endotoxin 24 hr. earlier
○ Controls - no prior endotoxin
△ Bilateral renal cortical necrosis present
LUNG
Received 0.25 mg. E. coli endotoxin 24 hr. earlier
Controls - no prior endotoxin
PER CENT RADIOACTIVITY INJECTED
HOURS AFTER INJECTION OF ENDOTOXIN
0 3 6 9 12 15 18 21 24

FIGS. 3, 4. PER CENT OF INJECTED RADIOACTIVITY IN THE KIDNEY (FIGURE 3) AND LUNG (FIGURE 4) AT VARIOUS TIMES AFTER AN INTRAVENOUS INJECTION OF $Cr^{51}$ LABELED ENDOTOXIN IN NORMAL AND PREPARED RABBITS

Each point represents the value for one animal.

## DISCUSSION

These experiments demonstrate significant alterations in localization of endotoxin in the organs of the rabbits prepared for the generalized Shwartzman reaction. Twenty-four hours after a sublethal intravenous injection of *E. coli* endotoxin, the capacity of the liver to take up radioactive $Cr^{51}Cl_3$ labeled endotoxin was reduced to approximately one-half normal. On the other hand, the uptake by the lung, and to a lesser extent by the spleen, was increased in the prepared rabbits. The relation of these shifts in localization of radioactive endotoxin to pathogenesis of the generalized Shwartzman reaction, however, is not clear. In general, prepared rabbits showed the altered pattern of localization regardless of whether or not renal cortical necrosis developed. Likewise, rabbits having glomerular fibrinoid lesions upon microscopic examination did not differ consistently in pulmonary or hepatic uptake from those not showing this lesion.

The only definite correlation between shifts of radioactivity and the lesions of the generalized Shwartzman reaction was found in the increased localization in kidneys showing renal cortical necrosis. Although this observation implies that increased localization of endotoxin in the kidney may result directly in cortical necrosis, another possible explanation seems as likely. Previous studies (6) have demonstrated that soon after injection into normal rabbits radioactive endotoxin localized in the "buffy coat" of the blood and presumably in the polymorphonuclear leucocytes. Since renal cortical necrosis is accompanied by infiltration of the swollen juxta-cortical areas with leucocytes (8), it seems possible that renal concentration of radioactivity may represent the trapping of endotoxin-laden leucocytes in the necrotic areas. This same mechanism might also account for greater uptake by the lung.

The decreased uptake of endotoxin in the liver of the prepared rabbits is of considerable interest. The concept that R.E. function of the liver is impaired by the preparing injection of endotoxin receives support from several previous studies. "Blockade" of the R.E. system (3, 4) brings about a state of preparation for the generalized Shwartzman reaction in rabbits which resembles, in every demonstrable way, that resulting from an injection of endotoxin. Cornwell (9) determined plasma clearance rates and organ uptake of radioactive colloidal gold in prepared rabbits in an attempt to assess the functional capacity of the R.E. system after an injection of endotoxin. He demonstrated a decreased plasma clearance and hepatic uptake in the prepared rabbit. Gabrielli (10) has reported similar results using radioactive colloidal chromium, and Biozzi, Benacerraf, and Halpern (11) recently demonstrated decreased hepatic uptake of injected ink particles after injection of typhoid vaccine.

Therefore, the available evidence indicating decreased capacity of the liver to take up endotoxin is compatible with the interpretation that an R.E. function of this organ is compromised in the "prepared" rabbit. It is conceivable, however, in view of the marked hemodynamic effects of endotoxin, especially involving splanchnic and hepatic blood flow (12), that other mechanisms may be in-

volved. Regardless of their interpretation the functional alterations of the prepared state revealed in these studies do not appear to bear a direct relationship to the microscopic fibrinoid lesions which characterize the generalized Shwartzman reaction. In this respect the data support the suggestion made previously (13, 14) that the acute lethal action of endotoxin and the fibrinoid vascular lesions in the Shwartzman reaction do not necessarily depend upon the same mechanisms.

## SUMMARY

Attempts have been made to characterize the prepared state in the generalized Shwartzman reaction by determining the fate of intravenously injected $Cr^{51}$ labeled radioactive endotoxin in rabbits 24 hours after a preparing injection of unlabeled endotoxin. Compared with normal rabbits, the prepared rabbit exhibited a markedly altered pattern of distribution of radioactivity. Hepatic uptake was reduced nearly 50 per cent and pulmonary uptake was increased. No increase in renal localization was observed except in those rabbits developing bilateral renal cortical necrosis. In animals not developing necrosis, no direct relationship was found between the renal or hepatic uptake of endotoxin and the microscopic fibrinoid lesions of the generalized Shwartzman reaction.

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# *IN VIVO* STUDIES OF THE ROLE OF ALBUMIN IN ENDOGENOUS AND HEPARIN-ACTIVATED LIPEMIA-CLEARING IN NEPHROTIC RATS[1]

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In a previous study (1) heparin was found to inhibit the rise of plasma lipids and cholesterol occurring in rats with nephrosis experimentally induced by injection of anti-rat kidney serum (AKS). Such inhibition was marked when heparin was administered prior to and immediately after the injection of AKS, but of much lesser degree when administered to the chronic nephrotic rat with established hypoalbuminemia and hyperlipemia. A similar slight or absent anti-lipemic response to heparin has been observed in some human subjects with nephrosis (2). This suggested to us that perhaps the inhibitory action of heparin necessitated the presence of a larger concentration of plasma albumin than was present in the chronic nephrotic rat.

Therefore a second study (3) was done in which bovine serum albumin was infused into rats during both the induction and chronic phases of experimental nephrosis. This was found to be far more effective than heparin in respectively preventing and correcting the hyperlipemia otherwise occurring in such rats. However, the infusion of albumin was not found capable of completely normalizing the plasma lipids of the nephrotic animal unless it concomitantly was nephrectomized.

This suggested that the accumulation of lipids in the plasma of the nephrotic rat is due not only to a quantitative deficiency of plasma albumin (3) but also to the excessive renal loss of some other essential plasma constituent of the lipid-clearing mechanism which might be escaping *pari passu*, either separately or combined with the escaping albumin. If this substance were heparin, or its plasma equivalent, the combined administration of heparin and albumin would be expected to be completely effective in normalizing the plasma lipids of the nephrotic rat. This was found to be true in the present study of such combined treatment, the data also providing an *in vivo* demonstration of the essential role of adequate plasma albumin for both endogenous and heparin-activated processes of lipemia-clearing.

[1] Aided by Grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, (A-46, C-4), the American Heart Association and the San Francisco Heart Association.

## METHODS AND RESULTS

Adult, male rats (Long-Evans) were used in these studies. Pooled rabbit anti-rat kidney serum (AKS) was prepared as described by Heymann and Lund (4) and assayed in preliminary studies by its ability to induce hypoalbuminemia, hyperlipemia and hypercholesteremia within 18 hours after its intravenous injection. Continuous intravenous infusions were administered by a special constant infusion apparatus designed to inject 0.25 ml. per hour (6.0 ml. per 24 hours) through an exteriorized polyethylene cannula placed in an inferior lumbar vein during a preliminary operation accomplished under ether anesthesia. The injected solutions were either freshly prepared aqueous bovine serum albumin (30 per cent) or sodium chloride (0.9 per cent), the latter being used since it was previously found to induce a degree of hemodilution in nephrotic rats comparable to that induced by the albumin solution (3). In certain instances 5 mg. of sodium heparin was added to each 6-ml. aliquot of infusion solution. Plasma total lipids, total cholesterol, and albumin were determined by the methods previously described (3).

### A. *The effect of albumin-heparin infusion on plasma lipids during the induction of the nephrotic state.*

Each of 50 rats was prepared for intravenous infusion and injected with AKS (1 ml.). During the ensuing 24 hours, nine rats (Group I) were continuously infused with six ml. saline-heparin solution, as described above, and nine rats (Group II) with albumin-heparin solution. For control purposes 19 rats (Group III) were infused with albumin solution and 13 rats (Group IV) with saline solution, without added heparin. All rats were bled for plasma total lipids, total cholesterol and albumin at the end of the 24-hour infusion interval, and hematocrits were determined in those infused with saline (Group IV) and albumin (Group III).

TABLE I

*The effect of continuous 24-hour infusion of albumin and heparin on plasma lipids of rats concomitantly injected with antikidney serum*

| Group | Infusion fluid | No. of rats | Aver. wt. (gm.) | Average plasma concentrations* | | | |
|---|---|---|---|---|---|---|---|
| | | | | Total cholesterol | | Total lipids concentration (mg./100 ml.) | Albumin (gm./100 ml.) |
| | | | | Concentration (mg./100 ml.) | Average increase† (mg./100 ml.) | | |
| | | | | *Rats injected with antikidney serum* | | | |
| I | Saline + Heparin | 9 | 161 | 125 | +63 | 318 | 1.1 |
| | Range: | | | (94–162) | (+32–100) | (219–365) | (0.9–1.7) |
| | S.E. Mean: | | | ±7.8 | ±7.7 | ±15.6 | ±0.12 |
| II | Bovine Albumin + Heparin | 9 | 160 | 65 | +3 | 254 | 4.0 |
| | Range: | | | (30–89) | (+27–(−32)) | (191–303) | (2.5–5.3) |
| | S.E. Mean: | | | ±6.9 | ±6.8 | ±14.5 | ±0.43 |
| III | Bovine Albumin | 19 | 158 | 108 | +46 | 297 | 3.9 |
| | Range: | | | (81–131) | (+19–69) | (238–349) | (3.1–5.1) |
| | S.E. Mean: | | | ±9.3 | ±2.9 | ±7.3 | ±0.68 |
| IV | Saline | 13 | 162 | 153 | +91 | 413 | 1.2 |
| | Range: | | | (117–187) | (+55–125) | (275–540) | (0.5–1.5) |
| | S.E. Mean: | | | ±6.3 | ±6.3 | ±15.4 | ±0.10 |
| | | | | *Control rats injected with normal rabbit serum* | | | |
| V | Saline | 10 | 164 | 62 | | 191 | 2.3 |
| | Range: | | | (52–70) | | (142–228) | (2.0–2.6) |
| | S.E. Mean: | | | ±2.2 | | ±6.2 | ±0.11 |

* At completion of continuous 24-hour infusion (6.0 ml. per rat).
† Increment in excess of 62 mg. per 100 ml. (Group V).

Control plasma concentrations were established by bleeding 10 other normal rats (Group V) 24 hours after injection of 1.0 ml. of *normal* rabbit serum and infusion of saline.

In Table I it can be seen that the injection of AKS induced in 24 hours in the control rats (Group IV) a fall of plasma albumin to an average of 1.2 gm. per 100 ml., with an associated average increase of plasma lipids to 413 mg. per 100 ml. and of plasma cholesterol to 153 mg. per 100 ml. The rats infused with heparin (Group I) exhibited a comparable fall of plasma albumin (average: 1.1 gm. per 100 ml.), but a significantly lesser rise of plasma lipids (average: 318 mg. per 100 ml.) and of cholesterol (average: 125 mg. per 100 ml.). Since the infusion of heparin did not affect the fall of plasma albumin in these rats, there is no evidence that the observed lipemia-inhibitory effects were due to any effect of heparin on the immune mechanism by which AKS induced nephrosis, as may possibly occur under certain experimental circumstances (1, 5).

The infusion of albumin (Group III) prevented the fall of plasma albumin (average: 3.9 gm. per 100 ml.) and, as observed previously (3), even more effectively inhibited the rise of plasma lipids (average: 297 mg. per 100 ml.) and of cholesterol (108 mg. per 100 ml.) otherwise induced by AKS injection. However, only the combined infusion of heparin and albumin (Group II) proved completely effective in inhibiting the AKS-induced rise of plasma lipids (average: 254 mg. per 100 ml.) and cholesterol (average: 65 mg. per 100 ml.). These rats exhibited an average plasma albumin of 4.0 mg. per 100 ml., similar to those infused only with albumin (Group III). Moreover, the observed results could not be ascribed to any significant difference of induced hemodilution since the average hematocrit was 44 (Range: 41 to 48) in the rats infused with saline (Group IV), and 40 (Range: 38 to 41) in the albumin-infused rats (Group III).

## B. *The effect of albumin-heparin infusion on the plasma lipids of chronic nephrotic rats*

The previous experiment indicated that heparin only incompletely inhibited the rise of plasma lipids during the induction of the nephrotic state, and even less effectively inhibited the rise of plasma cholesterol. Similarly, as observed previously (3), albumin infusion was not *completely* effective in inhibiting the rise of plasma lipid and cholesterol unless it was combined with heparin. In view of the apparent need of both substances in preventing the occurrence of AKS-induced hyperlipemia and hypercholesteremia, it appeared of importance to us to determine whether the combination of heparin and albumin also was necessary to correct an *already established* hyperlipemia and hypercholesteremia in chronic nephrotic rats.

Each of a series of 46 rats was injected with one ml. AKS. Four days later the rats were divided into four

TABLE II

*The effect of continuous 24-hour infusion of albumin and heparin on plasma lipids of chronic nephrotic rats four days after injection of antikidney serum*

| Group | Infusion fluid* | No. of rats | Aver. wt. (gm.) | Average plasma concentrations | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Pre-infusion | Post-infusion | | | |
| | | | | | Total cholesterol | | | |
| | | | | Total cholesterol (mg./100 ml.) | Concentration (mg./100 ml.) | Average change (mg./100 ml.) | Total lipids (mg./100 ml.) | Albumin (gm./100 ml.) |
| I | Saline + Heparin | 9 | 186 | 206 | 217 | +11 | 429 | 1.1 |
| | Range: | | | (163–226) | (157–290) | (+60–(−52)) | (262–568) | (0.8–1.4) |
| | S.E. Mean: | | | ±11.0 | ±10.4 | ±11.1 | ±20.6 | ±0.30 |
| II a) | Bovine Albumin + Heparin | 8 | 180 | 224 | 66 | −158 | 243 | 3.2 |
| | Range: | | | (168–283) | (55–82) | (−98–(−223)) | (186–302) | (2.0–4.7) |
| | S.E. Mean: | | | ±14.3 | ±3.2 | ±14.9 | ±9.6 | ±0.16 |
| b) | Bovine Albumin + Heparin | 4 | 170 | 279 | 348 | +69 | 581 | 1.0 |
| | Range: | | | (181–423) | (195–501) | (+8–221) | (415–954) | (0.7–1.2) |
| III | Bovine Albumin | 11 | 160 | 223 | 116 | −107 | 331 | 3.0 |
| | Range: | | | (167–278) | (54–218) | (−27–(−203)) | (240–510) | (2.3–4.4) |
| | S.E. Mean: | | | ±11.6 | ±15.0 | ±14.4 | ±26.6 | ±0.20 |
| IV | Saline | 14 | 159 | 221 | 220 | −1 | 550 | 1.1 |
| | Range: | | | (142–281) | (135–287) | (+48–(−44)) | (415–662) | (0.6–1.4) |
| | S.E. Mean: | | | ±15.5 | ±16.9 | +8.6 | ±17.7 | ±0.16 |

* Six ml. per 24 hours.

groups and prepared for intravenous infusion. During the ensuing 24 hours nine of the rats (Group I) were continuously infused with saline-heparin and 12 others (Group II) with albumin-heparin solution. The remaining rats served as controls, 11 (Group III) being infused only with albumin and 14 (Group IV) only with saline.

In order to obviate the effect of initial removal of any significant amount of blood, the rats were bled only for plasma total cholesterol preoperatively. On completion of the 24-hour infusion all rats again were bled for determination of plasma total lipids, cholesterol and albumin.

The results are shown in Table II. The rats infused with saline containing heparin (Group I) exhibited an average plasma albumin of 1.1 gm. per 100 ml. at the end of the infusion interval. During this interval no significant change occurred in their average plasma cholesterol, which rose an average of 31 mg. per 100 ml. in six of the rats and fell an average of 30 mg. per 100 ml. in the remaining three rats. The respective saline-infused controls (Group IV) exhibited a comparable average plasma albumin of 1.1 gm. per 100 ml. and, as in the above rats, the infusion of saline failed to alter the average plasma cholesterol, which rose an average of 29 mg. per 100 ml. in six and fell an average of 19 mg. per 100 ml. in eight of these rats. However, the average plasma total lipids of these two groups of rats was 429 mg. per 100 ml. and 550 mg. per 100 ml., respectively, indicating a moderate heparin-induced lowering of total plasma lipids. The combined infusion of heparin and albumin increased the plasma albumin content of only eight of the 12 rats so treated (Group II a), to an average of 3.2 gm. per 100 ml. This was associated with a marked lowering of the plasma lipids of these eight rats to an average of 243 mg. per 100 ml., with an associated marked fall of their plasma cholesterols to normal levels averaging 66 mg. per 100 ml. That this response could not be ascribed only to the anti-lipemic effect of the albumin infusion *per se* (3) was shown by comparison with the respective control rats infused only with albumin (Group III) and which exhibited a comparable rise of plasma albumin (average: 3.0 gm. per 100 ml.) during the experimental interval, but a lesser fall of plasma lipids to an average of 331 mg. per 100 ml., and of cholesterol to an average of 116 mg. per 100 ml. The essential nature of adequate albumin for heparin-induced lipemia-clearing was particularly well shown by the findings in the remaining four of the 12 rats similarly infused with combined albumin-heparin (Group II b) but in which there failed to develop a rise of plasma albumin (average: 1.0 gm. per 100 ml.) which exceeded those of the saline-infused controls. Thus, despite the infusion of heparin, these rats exhibited plasma lipid levels averaging 581 mg. per 100 ml., comparable to those of the saline-infused controls (Group IV), and a further rise of their plasma cholesterols from an average of 279 mg. per 100 ml. to an average of 348 mg. per 100 ml. occurred during the 24-hour experimental interval.

## DISCUSSION

Albumin has been shown (6–10) clearly to provide the vehicle for the transport of the fatty acids

released during the hydrolysis of triglyceride substrates, and a relative deficiency of albumin has been shown (6–8) *in vitro* to block such lipolysis. Since the hydrolysis of plasma triglycerides appears, moreover, to be a prerequisite for clearance of lipid from the plasma (11, 12), the retention of excess lipid in the plasma would be expected in certain states characterized by the rapid loss and sustained depletion of plasma albumin. Consonant with this is our earlier finding (13) that the nephrotic state in rats is characterized by an isolated accumulation of excess lipid which is confined to their plasma. The essential role of albumin for the maintenance of endogenous processes of lipolysis and lipid-clearing from the plasma was confirmed *in vivo* by our recent demonstration (3) of the causal relationship between hypoalbuminemia and hyperlipemia in the experimentally nephrotic rat. The albumin deficiency blocks lipolysis and consequently also the egress of lipid from the plasma was also confirmed in the present studies in which the infusion of albumin again was shown to induce the rapid clearance of excess lipid from the plasma of nephrotic rats exhibiting hypoalbuminemia and hyperlipemia.

The lipemia-clearing induced by heparin administration in nephrotic human subjects has been found to be of limited magnitude and occasionally even absent (2). A similar limited anti-lipemic response to heparin was observed previously by us (1) in chronic nephrotic rats. In the present studies it was shown that this limited ability of heparin to clear excess lipids from the nephrotic rat's plasma is due to the sustained deficiency of albumin in such plasma, confirming *in vitro* demonstrations (6–10) of the essentiality of adequate albumin for heparin-activated processes of lipolysis and lipemia-clearing. It was also shown that the rate of clearance of excess lipid from the nephrotic rat's plasma during the infusion of albumin was considerably augmented by the concomitant administration of heparin. This is consonant with the belief (14–19) that heparin-induced, lipemia-clearing is simply an acceleration of the normally occurring lipolytic processes. The observed response to heparin-albumin infusion also indicated the ability of the nephrotic rat to form potent lipemia-clearing factor and failed to confirm the presence in nephrotic rat's plasma of some "clearing-factor inhibitor," as has been suggested by others (20).

It is likely that the clearance of excess cholesterol from the nephrotic rat's plasma, following administration of albumin and/or heparin, is secondary to the effects of these substances on the hydrolysis of triglycerides (10), since in turn, the accumulation of excess cholesterol in nephrotic plasma is probably secondary to the hypertriglyceridemia induced by the albumin deficiency (3). Thus, in the normal rat (21), progressive hypercholesteremia occurs when a sustained rise of plasma triglycerides is induced by the latter's continuous intravenous infusion. Since a progressive rise of plasma cholesterol also occurs in normal rats that are continuously infused with phospholipids (22), the hypercholesteremia in nephrotic plasma may, in part, be secondary to the hyperphospholipemia which also occurs in the nephrotic state. It is likely (21, 23–25) that the increment of excess cholesterol and phospholipid in nephrotic plasma, as in other hyperlipemic states, is "dissolved" in the excess insoluble triglycerides present in such plasma. The clearing of excess cholesterol from the plasma of the nephrotic rats infused with albumin or albumin-heparin would thus appear to be secondary to the dissolution of triglycerides known to be accomplished by such treatment (10, 24, 25).

In the present studies, as observed previously (3), the infusion of albumin was not found capable either of completely preventing the rise of plasma lipids and cholesterol induced by injection of antikidney serum, or of completely correcting the hyperlipemia and hypercholesteremia of chronic nephrotic rats. However, since the combined administration of heparin and albumin was found completely effective in this regard, it is likely that this partial failure on the part of albumin administration was not due to the use of "prepared" bovine serum albumin, rather than more physiological rat serum albumin, or to the presence of some clearing-factor inhibitor in the nephrotic rat's plasma (20). On the other hand, when the chronic nephrotic rat was nephrectomized, and then infused with albumin (3), as complete a normalization of the plasma lipids occurred as was observed presently in intact nephrotic rats infused concomitantly with albumin and

heparin. This suggests that a causal factor in the occurrence of nephrotic hyperlipemia, other than the quantitative deficiency of plasma albumin (3), may be the renal loss of "clearing-factor" or some co-factor, occurring alone or in combination with escaping albumin. An additional factor would appear to be the short intravascular existence of any given albumin molecule. Thus, in certain of the treated chronic nephrotic rats, the renal loss of infused albumin occurred so rapidly (3, 26) as to preclude any rise of plasma albumin, and in this instance no lipemia-clearing was induced by the injected albumin or by the concurrently administered heparin.

SUMMARY

The effect of administration of heparin and albumin, alone or in combination, upon the plasma lipids of nephrotic rats was studied during both the induction and chronic phases of experimental nephrosis induced by antikidney serum injection. It was found that heparin administration induced only a minimal lipemia-clearing response in severely hypoalbuminemic rats, unless albumin was simultaneously administered. The concurrent administration of heparin markedly augmented the ability of infused albumin to correct the hyperlipemia and hypercholesteremia of the nephrotic rat. It was suggested that an additional causal factor underlying nephrotic hyperlipemia, other than deficiency of plasma albumin, may be the renal loss in such rats of "clearing-factor" or some co-factor essential to lipolytic processes.

The data provide an *in vivo* confirmation of the essential role of albumin for endogenous and heparin-activated lipemia-clearing and are consonant with the belief that heparin effects lipemia-clearing by accelerating normally occurring lipolytic processes.

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# BIOCHEMICAL OBSERVATIONS ON THE USE OF L-GLUTAMIC ACID IN THE TREATMENT OF HEPATIC COMA

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The neurological pattern of hepatic coma has been characterized by Adams and Foley (1), and their observations facilitate the early recognition of this syndrome. A similar syndrome has been produced by the administration of certain nitrogenous substances (2) or a high protein diet (3) and has been observed following portacaval shunt (4) or total hepatectomy (5). Blood ammonia levels in hepatic coma are usually elevated, but there is poor correlation with the neurological state. Walshe (6) reported in 1953 the successful treatment of hepatic coma with large amounts of l-glutamic acid. Although beneficial results have been reported with glutamic acid by some investigators (7–11), in contrast, no benefit has been observed from this treatment by others (12–14). However, independent of the clinical result the blood ammonia decreases with glutamic acid therapy (7, 12, 14).

Walshe (6) proposed that an excess of intracellular ammonia accumulates in the brain in hepatic coma because of disturbed metabolism of glutamic acid. The administration of glutamic acid might correct this defect. Bessman and Bessman (15) believed that interference with energy metabolism in the brain by removal of alpha-ketoglutarate is the essential lesion of hepatic coma. The present study characterizes some of the biochemical changes in the serum accompanying the fall in blood ammonia produced by the infusion of sodium glutamate in normal persons and patients in hepatic coma.

## CLINICAL PROCEDURES AND METHODS

Glutamic acid infusions were given to two normal subjects and eight patients with cirrhosis. All were studied on the wards of the Walter Reed Army hospital. The diagnosis of pre-coma was made in such cases by serial observations of developing stupor, confusion, and flapping tremor in patients with chronic liver disease. The criteria for the definition of coma are those outlined by Webster and Davidson (13). All patients were fasting during the time of study and glucose was administered at a constant rate as far as possible. None of the patients was in shock, but patients 8, 9, and 4 during Study "C" received blood transfusion during the aftercontrol period because of continued gastrointestinal hemorrhage. Azotemia was not observed but oliguria was present in all coma patients. Pertinent clinical information for each subject is given in Table I.

Blood ammonia was determined on fresh heparinized venous blood. All determinations were made in triplicate using a microdiffusion method (16, 17). The average deviation of replicates on the same blood sample from the mean (18) was $7.6 \pm 3.1$ $\mu$M per L.[3] Simultaneous arterial and venous blood samples were obtained from five patients and analyzed for ammonia and alpha-ketoglutarate. The arterial blood ammonia was always higher than the venous blood ammonia, suggesting extraction of ammonia by the musculature as reported by Bessman and Bradley (19), but the alpha-ketoglutarate did not change. The impression was gained during the collection of samples that the brighter venous bloods had higher ammonia values. For this reason, the effects of *in vitro* oxygenation on the blood ammonia were studied. Venous blood was equilibrated in a tonometer with a stream of washed oxygen or nitrogen, and at the end of five minutes the original unagitated venous blood and both treated samples were analyzed for ammonia. The nitrogen equilibration was employed as a control, for the gassing process caused the loss of some ammonia. All determinations were performed in triplicate and the sequence of analysis was varied so that the oxygenated sometimes preceded and sometimes followed the other analyses. Actual ammonia analyses were started within 20 minutes of shedding of the blood and the first and last samples were invariably less than five minutes apart. The oxygenated blood assumed a bright red color in contrast to both the control venous and the nitrogenated blood; however, the oxygen tension was not directly measured. Blood from six normal persons and from four patients with cirrhosis was treated in this manner.

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[3] The designation $\mu$M per L. is used for all substances to facilitate comparisons. One $\mu$M per L. of ammonia is equivalent to 0.017 $\gamma$ per ml.

TABLE I

*Pertinent clinical information on the normal subjects and patients with cirrhosis*

| No. | Age | Diagnosis | Duration of cirrhosis *months*† | Total bilirubin at time of study *mg./100 ml.* | Serum albumin *gms. per 100 ml.* | Factor precipitating coma | Duration of coma before glutamate infusion *hrs.* | Effect of glutamate infusion | Comment |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 21 | Normal | None | 0.5 | 5.0 | | | None | |
| 2 | 26 | Normal | None | 0.3 | 4.6 | | | None | |
| 3 | 43 | *Cirrhosis | 36 | 1.4 | 4.1 | | | None | |
| 4a | 62 | Cirrhosis | 18 | 32 | 3.4 | Hemorrhage | 6 | None | Died 4 days later |
| b | | | | | | | 30 | None | |
| c | | | | | | | 54 | None | |
| 5 | 44 | Cirrhosis | ½ | 19 | 2.8 | ?Paraldehyde | 36 | Awakened | Died after 12 days |
| 6 | 46 | Cirrhosis | 4 | 3.2 | 2.2 | ?$NH_4Cl$ Hemorrhage | 6 | None | Precoma due to $NH_4Cl$? Died 24 hrs. later |
| 7a | 45 | Chronic pancreatitis; cirrhosis | 2 | 28 | 2.2 | | 24 | None | Coma lightened |
| b | | | | | | | 12 | None | Coma increased; death 36 hrs. later |
| 8 | 45 | Cirrhosis | 13 | 35 | 2.0 | ?Transfusion reaction | 36 | None | Died 24 hrs. later |
| 9 | 51 | Cirrhosis | 66 | 9 | 1.7 | Hemorrhage | 8 | ?Convulsions | Died 6 hrs. later |
| 10 | 52 | Non-alcoholic cirrhosis | 8 | 4.4 | 1.4 | ?I.V. protein hydrolysates | 24 | None | Died 15 days later |

* No signs of coma.
† Dated from the time of the first clinical diagnosis or unequivocal historical sign of decompensation.

None of the patients is one of those described in this paper, for this phenomenon was not investigated at that time. The results are recorded in Table II. The blood saturated with oxygen from both the normal and cirrhotic patients had a higher ammonia value than blood saturated with nitrogen, and the control value was generally intermediate. The interpretation of arterial-venous ammonia differences remains difficult until the *in vitro* effects of oxygenation, recently confirmed (20), are further clarified.

Glutamine was measured by the procedure of Iber and Bruton (21), employing an enzyme prepared from the kidneys of acidotic rats. Pyruvic acid and alpha-ketoglutarate were measured by the method of Seligson, McCormick, and Sborov (22). Citric acid was measured by the method of Ettinger, Goldbaum, and Smith (23). Amino acids were measured by column chromatography using the technique of Moore and Stein[4] (24). A portion of the glutamine is hydrolyzed on the column in this method and appears as glutamic acid.

Monosodium glutamate was infused into two normal persons and eight patients with cirrhosis. All patients received intravenous glucose before and after study. Monosodium glutamate (approx. 24 gm.) was prepared in 500 cc. of 5 per cent glucose; the rate of infusion was adjusted so that glucose was given at the same rate before, during, and after the glutamate infusion. The time, amount, and duration of the glutamate infusion are given in Table III. Blood samples were taken in aqueous heparin and processed immediately for analysis. The samples for citric acid, pyruvic acid, alpha-ketoglutaric acid, glutamine, and amino acids were stored frozen until analyzed.

[4] These determinations were obtained through the cooperation of Dr. Stanley Levenson and Mr. Hyman Rosen.

## RESULTS

### *Blood "ammonia"*

The blood "ammonia" was elevated prior to therapy in the patients in coma (average value, 215 μM per L.; normals, 67.6) (Table III), but

TABLE II

*The effects of oxygen tension on blood "ammonia" (gamma per ml.)**

| | | | | | | |
|---|---|---|---|---|---|---|
| **Normals** | | | | | | |
| Oxygenation | 1.51 | 1.19 | 1.33 | 1.16 | 1.35 | 1.14 |
| Control Venous | 1.21 | 0.90 | 1.66 | 1.19 | 1.35 | 1.01 |
| Nitrogenation | 1.16 | 0.70 | 1.12 | 0.73 | 1.07 | 1.21 |
| $O_2-N_2$ Difference | 0.35 | 0.49 | 0.21 | 0.43 | 0.28 | −0.07 |
| **Cirrhotics** | | | | | | |
| Oxygenation | | 1.03 | 2.08 | 1.42 | 1.67 | |
| Control Venous | | 1.13 | 1.89 | 1.29 | 1.31 | |
| Nitrogenation | | 0.91 | 1.54 | 1.31 | 1.23 | |
| $O_2-N_2$ Difference | | 0.12 | 0.54 | 0.11 | 0.44 | |

* Mean difference of oxygenation—nitrogenation is 0.29/ml.
S.E. of difference is 0.062 $P < .01$.

TABLE III

*Changes in blood levels before and after intravenous sodium glutamate*

| No. | Dose of glutamate and duration | Control and duration | Ammonia μM./L. Before | Ammonia μM./L. After | Glucose mg./100 ml. Before | Glucose mg./100 ml. After | Alpha-keto-glutarate μM./L. Before | Alpha-keto-glutarate μM./L. After | Pyruvate μM./L. Before | Pyruvate μM./L. After | Citrate μM./L. Before | Citrate μM./L. After | Glutamine μM./L. Before | Glutamine μM./L. After |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal values* | | | 67.6 ± 10 | | | | 13.0 ± 1.5 | | 73 ± 16 | | 79.2 | | 550–760 | |
| 1† | 24 gm. 3½ hrs. | Glucose 2 hrs. | 166 | 72 | 88 | 96 | 12.4 | 20.6 | 115 | 128 | 70 | 58 | 229 | 265 |
| 2† | 22 gm. 3½ hrs. | Glucose 1½ hrs. | | | 77 | 82 | 11.3 | 17.8 | 69 | 71 | | | | |
| 3‡ | 24 gm. 3 hrs. | Glucose 2½ hrs. | 204 | 161 | | | 15.6 | 21.5 | 68.5 | 59.6 | 84.3 | 90.6 | | |
| 4a | 24 gm. 2½ hrs. | | 140 | 88 | 90 | 123 | 29.7 | 144.5 | 156 | 260 | 73.4 | 122 | 700 | 1,240 |
| b | 24 gm. 4½ hrs. | | 138 | 107 | | | | | | | | | | |
| c | 20 gm. 2 hrs. | Glucose 2 hrs. | 176 | 126 | | | | | | | | | 516 | 410 |
| 5 | 32 gm. 4.5 hrs. | | 148 | 76 | | | 53.3 | 76.5 | 224.4 | 106.1 | 127 | 114 | | |
| 6 | 20 gm. 5.5 hrs. | Glucose 2 hrs. | 370 | 122 | 53 | 65 | 36.8 | 57.5 | 355 | 468 | 368 | 290 | | |
| 7a | 18 gm. 6½ hrs. | | 165 | 103 | 124 | 212 | 33.2 | 47.5 | 56 | 103 | 201 | 223 | | |
| b | 24 gr. 3 hrs. | Ca gluconate 2 hrs. | 248 | 149 | 114 | 107 | 65.1 | 83.0 | 326 | 259 | 130 | 102 | 1,510 | 1,580 |
| 8 | 24 gm. 3 hrs. | | 2,440 | 3,550 | 90 | 69 | 36.2 | 117 | 506 | 387 | 154 | 198 | 1,630 | 1,610 |
| 9 | 40 gm. 3 hrs. | Glucose 3 hrs. | 421 | 254 | 133 | 160 | 83.0 | 60.3 | 517 | 433 | 103 | 108 | | |
| 10 | 20 gm. 3½ hrs. | Glucose 2 hrs. | 191 | 158 | 208 | 178 | 23.7 | 18.8 | 123 | 108 | | | 925 | 990 |

* From the authors' laboratory. Glutamine range based on ten normals (21), mean citrate value taken from Ettinger, Goldbaum, and Smith (23). Pyruvate and alpha-ketoglutarate based on 17 normal subjects and the ammonia on 25 normal subjects.

† "Normal" patients.

‡ Patient with cirrhosis and no signs of coma.

the degree of elevation did not correlate with the depth of the coma. The levels tended to rise during the control infusions of glucose. In patient 8 extremely high levels of ammonia were found, 2,440 μM per L. initially, and values of similar magnitude were found in three subsequent blood specimens. The patient died a few hours after these observations. Review of the clinical information led subsequently to the suspicion that this man had septicemia with a urea splitting organism, but no blood culture was taken while the patient was alive and review of the pathology sections for bacteria did not support this possibility. The ammonia values of this case are excluded from the averages and from the figures in this paper. Sodium glutamate was effective in lowering the blood ammonia in all other patients (Figure 1). The lowering of the blood ammonia was on the average 87 μM per L. or 40 per cent of the initial value. The degree of lowering was not related in a simple fashion to either the dose given or the rate at which it was given within the ranges employed. There was no relationship between the amount of lowering and the clinical changes in

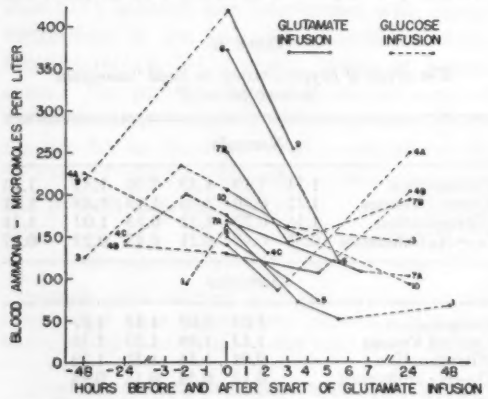


FIG. 1. THE EFFECT OF GLUTAMATE INFUSION ON THE CONCENTRATION OF BLOOD "AMMONIA"

The numbers identify the patients in Tables I and III.

the patient. Most of the ammonia values 24 hours after treatment had returned to pre-treatment levels.

*Alpha-ketoglutarate*

Blood alpha-ketoglutarate levels prior to the glutamic acid infusion were elevated from two to six times the normal level in the patients with liver coma. In four patients there was no striking change during the control infusions of glucose. In eight of ten infusions of glutamic acid there was a rise in ketoglutarate by from one-quarter to five times the initial value (Figure 2); in patients 9 and 10 there was a 25 per cent fall, possibly associated with a greater interval between cessation of the infusion and withdrawal of the specimen. In the study of patient 2, a normal subject, samples of blood were taken five minutes before the completion of the glutamic acid infusion, five minutes, 15 minutes, and 60 minutes after the completion. These data are presented in Table IV and Figure 3. The alpha-ketoglutarate level rises by 57 per cent and returns to the starting level within 15 minutes of the end of the infusion. The rapid changes in the levels of glutamic acid, alanine, and the glutamine-asparagine-serine complex are also to be noted.

*Pyruvate, citrate, and glutamine*

Blood pyruvate concentrations were elevated to two to seven times the normal in seven of eight of the comatose patients. Citrate concentration was elevated in six out of seven instances. The changes produced by glutamate infusion were variable. Glutamine was determined before and after six of the glutamate infusions. Although there was a rise in glutamine in four of the six infusions, only three of these showed a rise equal in concentration to the fall in ammonia. The

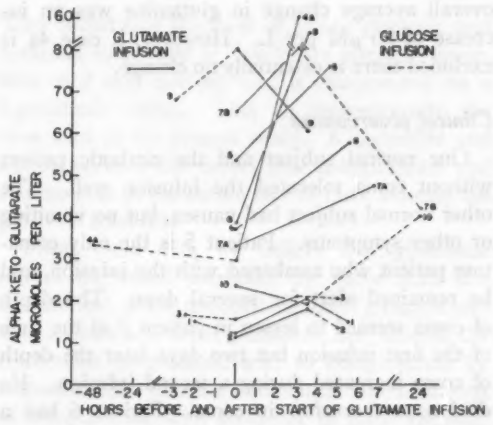
GLUTAMATE INFUSION
GLUCOSE INFUSION
ALPHA-KETO-GLUTARATE MICROMOLES PER LITER
HOURS BEFORE AND AFTER START OF GLUTAMATE INFUSION

FIG. 2. THE EFFECT OF GLUTAMATE INFUSION ON THE CONCENTRATION OF ALPHA-KETOGLUTARATE

The numbers identify the patients in Tables I and III.

TABLE IV

*Effect of sodium glutamate infusion on plasma substances* *

| μM./L. Plasma | Start of infusion | 5 Min. before end | 5 Min. after end | 15 Min. after end | 60 Min. after end |
|---|---|---|---|---|---|
| Threonine | 79 | 78 | 73 | 76 | |
| Glutamine, asparagine, serine † | 168 | 210 | 215 | 87 | |
| Glutamic acid | 112 | 1130 | 775 | 400 | |
| Glycine | 82 | 100 | 89 | 57 | |
| Alanine | 200 | 305 | 235 | 182 | |
| Ketoglutarate | 11.3 | 17.8 | 16.0 | 11.8 | 13.6 |
| Pyruvate | 69.1 | 71.0 | 67.5 | 78.8 | 70.2 |
| Citrate | 80 | 110 | 127 | 109 | 193 |
| Glucose | 77 | 82 | 83 | 80 | 85 |

* Twenty-four gm. Na glutamate given over 205 minutes to a normal subject. Glucose given at uniform rate throughout the study.

† These came off as a group on the ion-exchange column. There may be loss of glutamine.

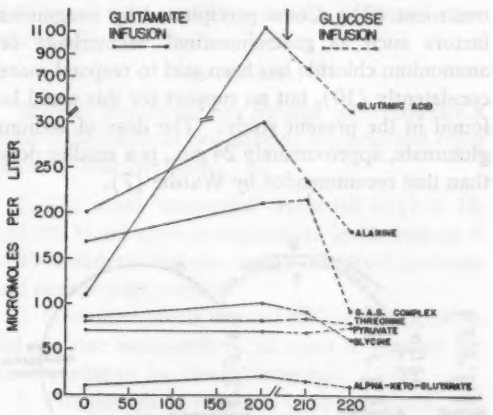


FIG. 3. CHANGES IN THE CONCENTRATIONS OF RELATED METABOLITES WITH TIME AFTER CESSATION OF GLUTAMATE INFUSION

Note the expansion of time scale after the cessation of glutamate. A 50 per cent rise and fall of ketoglutarate are minimized by the scale.

overall average change in glutamine was an increase of 96 μM per L. However, if case 4a is excluded there is essentially no change.

*Clinical observations*

One normal subject and the cirrhotic patient without coma tolerated the infusion well. The other normal subject had nausea, but no vomiting or other symptoms. Patient 5 is the only comatose patient who awakened with the infusion, and he remained alert for several days. The depth of coma seemed to lessen in patient 7 at the time of the first infusion but two days later the depth of coma increased during a second infusion. He died soon thereafter in coma. Patient 6 had a spontaneous remission from coma. Three days later coma recurred, and glutamic acid was given but did not produce a remission.

## DISCUSSION

Glutamic acid as employed in this study was of little value in the treatment of the clinical manifestations of hepatic coma. These observations confirm the experience of others (13, 14) who found only transient beneficial effects. It must be pointed out that our group of patients all had chronic liver disease underlying their coma, and that this group is most refractory to glutamic acid treatment (7). Coma precipitated by exogenous factors such as gastrointestinal hemorrhage or ammonium chloride has been said to respond more consistently (10), but no support for this could be found in the present study. The dose of sodium glutamate, approximately 24 gm., is a smaller dose than that recommended by Walshe (7).

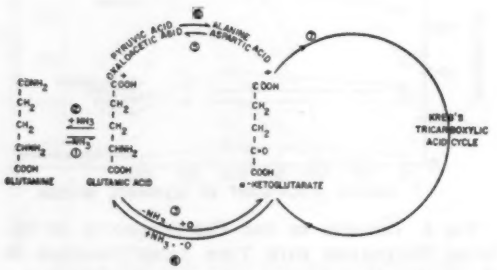


FIG. 4. SCHEMATIC INTERRELATIONS OF METABOLITES ARRANGED TO SUGGEST VARIOUS MECHANISMS BY WHICH GLUTAMATE MAY LOWER THE BLOOD "AMMONIA" CONCENTRATION

Before the biochemical changes are discussed two limitations of this study must be stated. All measurements were made on the blood, which reflects only gross changes in the body. The changes to be described for various metabolites probably occur at the intracellular level or in fluid compartments not in immediate equilibrium with the plasma. In addition, the data shown in Table IV and Figure 3 suggest that rapid changes in the blood levels at the time of cessation of the infusion may cause an appreciable variability in the data obtained from the rest of the patients.

The blood ammonia was lowered by glutamic acid in all except patient 8. Thus glutamic acid offers an effective method for lowering the blood ammonia. The possible reactions that could account for the disappearance of these quantities of blood "ammonia" (average 87 μM per L.) are outlined in Figure 4. The only direct reaction between glutamic acid and ammonia is No. 2, in which ammonia is added to glutamic acid to yield glutamine. Foster, Schoenheimer, and Rittenberg (25) have observed that the disappearance of isotopically labelled $NH_3$ in the normal rat is accompanied by the rapid accumulation of radioactivity in amide nitrogen, presumably glutamine. The possible value of this reaction for the removal of ammonia initially led Walshe to infuse glutamic acid (6). Glutamine rose in four of the six cases in which it was measured but in only three was the magnitude sufficient to account for the fall in ammonia. Since glutamine is not known to transfer the ammonia to other substances, its accumulation might be expected. Conversions of glutamate to glutamine cannot be the sole mechanism for ammonia removal because the glutamine actually fell in cases 4c and 8 and rose inadequately in cases 1 and 7b. Bessman (26) believes that this reasoning is incorrect because the kidney deaminates glutamine so rapidly that it cannot accumulate in the blood. However, a lack of increase in glutamine after glutamic acid administration was also demonstrated by Walshe (7) and Singh, Barclay, and Cooke (12).

The route of removal of infused glutamic acid is unknown. The dose used in this study (24 gm.) would represent 2,300 μM per L. in 55 liters, the approximate total body water of patient 2, a man weighing 82 kg. At the end of the infusion the maximum level of glutamic acid was 1,130 μM

per L. and the level fell to less than half of this value 15 minutes after cessation of the infusion (Table IV, Figure 3). At no time were there rises in the glutamine to account for disappearance of this magnitude of glutamic acid. In patient 1 approximately 5 per cent of the infused glutamate appeared in the urine as an increase in alpha amino nitrogen excretion.

The rise in ketoglutarate in eight of the ten cases seemed directly related to the infusion of glutamate (Figure 2). There are two principal routes by which glutamic acid can give rise to ketoglutarate (Figure 4), reaction No. 3 in which ammonia is released, and reaction No. 6, the transamination reaction, in which one molecule of glutamic acid reacts with the keto-acids pyruvic or oxaloacetic to yield, respectively, alanine or aspartic acid and one molecule of ketoglutarate. The latter reaction does not produce ammonia.

The observations in Table IV show that alanine increases after sodium glutamate infusion. This amino acid was measured before and after sodium glutamate infusion in one additional normal person and in three patients in hepatic coma, and it consistently increased in all after the infusion. The details of these observations are reported in a subsequent paper (27). The loss of glutamic acid and the gain of alanine by the plasma suggest that transamination (No. 6, Figure 4) is occurring. Pyruvic acid would therefore be expected to disappear at the same time. This disappearance was not observed, but glucose was being infused rapidly (approximately 700 $\mu$M per min.) in these patients and would serve to maintain the pyruvate level. The transamination reaction would also produce ketoglutarate. The ketoglutarate, once formed, may return to glutamic acid by reaction No. 4 and in this process would remove a molecule of free ammonia.

Walshe (7) presented data showing changes in ketoglutarate following glutamate infusions which are similar to these reported here, though he did not consider the rise in ketoglutarate significant. Pyruvic acid and citric acid levels were high in our study and thus confirm the observations that both are high in patients with liver disease (22, 28). However, the levels did not show consistent changes after the infusion of glutamate. Klingmüller and associates (29, 30) studied the disappearance of infused glutamic acid in rabbits. They found that the level of serum glutamic acid returned to normal in two hours after the injection of 2 mM per kg. and it disappeared on a logarithmic curve. This is approximately the dose used in the present study. Klingmüller and his co-workers also found that ketoglutarate and alanine rose in the serum and in their animals the pyruvate level fell.

From these data we believe that glutamate lowers the blood ammonia by more than one mechanism: First, the combination of glutamic acid with ammonia to form glutamine, and second, the conversion of glutamic acid to ketoglutarate by transamination and the subsequent recombination of ketoglutarate with ammonia to form glutamic acid. This conclusion is based to a large degree on our inability to demonstrate a rise in glutamine comparable in each case to the fall in blood ammonia. The glutamine method employed, though not specific for glutamine, would be expected to produce results that are correct or erroneously high, but never erroneously low. The suggestion of Bessman (26) that the kidney was rapidly removing the glutamine by the formation of urinary ammonia was not investigated.

The biochemical changes observed following an infusion of glutamic acid were similar in the normal subjects and the patients with liver disease. Additional data on this point are presented elsewhere (27).

## SUMMARY

Sodium glutamate was infused intravenously a total of 13 times in two normal subjects and eight patients with cirrhosis.

1. The blood "ammonia" levels fell in 11 of 12, and the blood alpha-ketoglutarate levels rose in 8 of 10 instances, and the changes in blood pyruvate and citrate were variable.

2. Glutamine levels showed variable changes but did not rise sufficiently in all cases to account for the lowering of the blood "ammonia."

3. The pattern of response to the glutamic acid infusion was similar for normal subjects, patients with cirrhosis, and patients with hepatic coma, although the initial levels of these substances were elevated in the patients with cirrhosis.

4. Only one of seven patients in hepatic coma showed a suggestive clinical response to sodium glutamate infusion.

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# ULTRACENTRIFUGAL CHARACTERIZATION OF THE LIPOPROTEIN SPECTRUM IN OBSTRUCTIVE JAUNDICE: STUDIES OF SERUM LIPID RELATIONSHIPS IN INTRA- AND EXTRAHEPATIC BILIARY OBSTRUCTION [1]

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The alterations in serum lipids accompanying obstructive jaundice have long been a matter of considerable interest. In the absence of significant hepatic failure, biliary obstruction of either intra- or extrahepatic type often results in markedly increased serum total cholesterol and phospholipid levels. The increase in serum phospholipid concentration may be relatively greater, resulting in an increased phospholipid:total cholesterol ratio. Since the increment in serum total cholesterol is due mainly to increase in the unesterified cholesterol fraction, the ratio phospholipid:free cholesterol may be more meaningful. This ratio, according to Jackson, Wilkinson, Hand, Waldron, and Vogel (1), has a minimal limiting value (by weight) of 2, suggesting a homeostatic mechanism operating to maintain a molar ratio of at least one phospholipid molecule per molecule of unesterified plasma cholesterol.

With the development of techniques such as ultracentrifugal flotation, paper and zone electrophoresis and plasma protein fractionation, the serum lipids and lipoproteins in biliary obstruction have again come under scrutiny. The serum lipid distribution in biliary obstruction has been studied by Barr, Russ, Eder, and their co-workers (2–4) employing Cohn method-10 (5) for lipoprotein fractionation. They found increased amounts of cholesterol in fractions IV + V + VI ("alpha lipoproteins"), mostly in the free form, and increased amounts of phospholipids in fractions I + III ("beta lipoproteins"). Kunkel and Slater (6), utilizing zone electrophoresis, noted marked reduction in alpha lipoprotein in biliary cirrhosis, together with increased amounts of phospholipid-rich lipid having the same relative mobility as normal beta lipoprotein. Partial ultracentrifugal characterization of the serum lipoproteins in obstructive jaundice by McGinley, Jones, and Gofman (7) indicated an increase in $S_f$ 0–10 and 10–20 lipoproteins, usually with a rather sharp cut-off in the analytical pattern at $S_f$ 17. The present authors extended the centrifugal characterization of the serum lipoproteins in obstructive jaundice by employing a solvent density of 1.21 gm. per L. (8). Markedly reduced or absent high density ($> 1.063$) lipoproteins and few, if any, low density lipoproteins of flotation rate greater than $-S_{1.21}$ 40–70 ($S_f$ 10–20) were reported to characterize the serum lipoprotein spectrum in biliary obstruction. This centrifugal characterization was subsequently confirmed and amplified by other workers (3, 9, 10).

We wish to report additional observations in 28 patients with biliary obstruction, particularly with respect to correlations found to exist between esterified cholesterol and high density lipoprotein concentrations.

## SUBJECTS AND METHODS

Patients with jaundice were selected for this study primarily from the hospitals of Oklahoma City. In most instances the etiology of the jaundice was clearly established by clinical course, biopsy or autopsy. When practicable, convalescent sera were obtained. Those subjects in whom exact diagnoses were not possible are listed as "unclassified jaundice," and the probable diagnosis is indicated.

[1] This work was supported in part by research grants from the National Heart Institute, National Institutes of Health, U. S. Public Health Service (H-1429 and H-1694), the Smith, Kline and French Foundation, and the Oklahoma State Heart Association.

[2] Dr. Conrad's work was done during the tenure of an American Heart Association Research Fellowship.

| Type | No. cases |
|---|---|
| [3]Pericholangiolitic biliary cirrhosis (11, 12) | 2 |
| Biliary cirrhosis secondary to extrahepatic obstruction | 2 |
| Common duct obstruction due to a variety of lesions | 6 |
| Primary hepatoma | 4 |
| Metastatic Ca of liver, primary breast | 2 |
| Cholecystitis and cholangitis | 1 |
| Infectious hepatitis | 2 |
| Homologous serum hepatitis | 3 |
| Unclassified | 6 |
| Total | 28 |

The van den Bergh reaction was utilized as a measure of serum bilirubin (upper normal values, total: 0.7 mg. per cent, direct: 0.4 mg. per cent) which, together with the alkaline phosphatase level (normal 2.0 to 4.5 Bodansky units per 100 ml.) provided an index of the degree of biliary obstruction. It is recognized that, in those subjects with metastatic carcinoma, some of the elevation in alkaline phosphatase levels might be due to the presence of osteoblastic activity resulting from osseous metastases. Cephalin flocculation (normal: up to 2+ in 48 hours) and thymol turbidity (normal: 0 to 4 units per 100 ml.) provided a rough index of the extent of hepatic functional impairment.

Post-absorptive serum was used for all lipid and lipoprotein determinations. Preparative ultracentrifugation was carried out according to the method of Green, Lewis, and Page (13, 14), in the Spinco Model L ultracentrifuge at 17° centigrade for 13 hours utilizing the 30.2 rotor at 30,000 r.p.m. (79,240 × gravity), solvent density 1.21 g. per ml.[4] For preparative centrifugation 2.00 grams of finely divided KBr were placed in each 10.5-ml. lusteroid centrifuge tube, 3.00 ml. of a 23.2 per cent solution of NaCl in water and 5.0 ml. of serum added.

Analytical centrifugation was accomplished in the Model E ultracentrifuge utilizing an "A" rotor at 52,640 r.p.m. (215,000 × gravity) 26.0 ± 0.5° centigrade. Analytical ultracentrifugation was sometimes repeated at a lower concentration, in order to provide $-S_{1.21}$ 25–40 ($S_f$ 0–12) and 40–70 ($S_f$ 12–20) lipoprotein patterns free of obscuring refractive bands. All planimetry in this study was done in triplicate by the same observer. High density $-S_{1.21}$ 0–12 lipoprotein peak areas were usually determined in the patterns obtained after centrifugation at a five-fold concentration (5 $C_0$) and the beta lipoprotein peak areas after centrifugation at 2 $C_0$ or 3 $C_0$. This permitted planimetry of maximum peak areas at the same time eliminating the need for extrapolation or other means of approximation. Strict attention was not paid to minor variations in flotation rates of the major lipoproteins nor was correction for Johnston-Ogston effects attempted. Conversion of area in square millimeters to milligrams per cent lipoprotein was accomplished utilizing formulation presented at the Technical Symposium of the Technical Group, Committee on Lipoproteins and Atherosclerosis, National Advisory Heart Council, 1952.

Total and unesterified cholesterol were determined essentially by the method of Sperry and Webb (16) and lipid phosphorus by a modification (17) of the method of Youngburg and Youngburg (18).

## RESULTS

It was deemed necessary to provide an index of the reduction in the high density $-S_{1.21}$ 0–12 lipoprotein concentration accompanying biliary obstruction which would have meaning over a wide range of serum cholesterol values, and the values of the ratios $-S_{1.21}$ 0–12 lipoprotein:total cholesterol and $-S_{1.21}$ 0–12 lipoprotein:free cholesterol were selected for this purpose. The values of these two ratios closely approach each other as the esterified serum cholesterol fraction falls to very low levels. The values of these ratios in jaundiced subjects were then compared to those obtained from 36 and 26 sets of observations on 16 and 14 healthy male subjects, respectively, ranging in age from 20 to 72. Studies from this laboratory and from Lewis and Page (19) indicate that, in healthy subjects, the lowest $-S_{1.21}$ 0–12 lipoprotein concentrations for any given serum cholesterol concentration are seen in adult males. Since the values for the ratios $-S_{1.21}$ 0–12 lipoprotein:total cholesterol and $-S_{1.21}$ 0–12 lipoprotein:free cholesterol were not normally distributed, but evidenced marked skewing to the left, the distributions were normalized by logarithmic transformation. The mean value of the ratio $-S_{1.21}$ 0–12 lipoprotein:total cholesterol, plus or minus two standard deviations, included all but

[3] We are indebted to Dr. H. E. MacMahon, Department of Pathology, Tufts College Medical School, for review of liver biopsies and verification of the diagnosis in these two patients.

[4] It has been suggested that this method of preparative centrifugation fails to float all the cholesterol (9). Analyses of infranatant fractions obtained in the authors' laboratory after preparative centrifugation by this technique have revealed a mean of 2.6 ± 0.8 per cent of the total native serum cholesterol concentration in 72 sera from healthy subjects, and a mean of only 0.3 ± 0.3 per cent in 63 infranatant fractions in biliary obstruction (15). These observations have been extended and the adequacy and reproducibility of the removal of the top fraction by pipetting has been compared with a tube slicing method. We are satisfied that this method of preparative centrifugation is adequate and reproducible. The explanation for the higher lipoprotein values reported by Havel, Eder, and Bragdon (9) utilizing a cholesterol-concentration product is outside the parameters of this paper.

one (94 per cent), *i.e.*, the lowest of the 36 values for this ratio in the 16 healthy male subjects. The mean value less two standard deviations, namely 0.731, was then selected as the value below which the $-S_{1.21}$ 0–12 lipoprotein:total cholesterol ratio of a patient must fall in order to be considered abnormally low. The mean value of the ratio $-S_{1.21}$ 0–12 lipoprotein:free cholesterol, plus or minus two standard deviations, included all (100 per cent) of the 26 values obtained in the 14 healthy male subjects. The lowest value for this ratio observed in the normal subjects, namely 2.63, was therefore selected as the level below which the value of the ratio $-S_{1.21}$ 0–12 lipoprotein:free cholesterol in a patient must fall to be considered abnormal.

The table contains the pertinent clinical and laboratory data resulting from this study. Inspection of the data reveals that moderate to marked reduction of high density lipoprotein concentrations, evidenced by $-S_{1.21}$ 0–12 lipoprotein: total cholesterol and $-S_{1.21}$ 0–12 lipoprotein:free cholesterol ratios below 0.73 and 2.63, respectively, characterize obstructive jaundice of many different etiologies. In the two subjects with pericholangiolitic biliary cirrhosis (DP and CD) no evidence of $-S_{1.21}$ 0–12 lipoproteins was detected in more than 100 sera. The $-S_{1.21}$ 0–12 lipo-

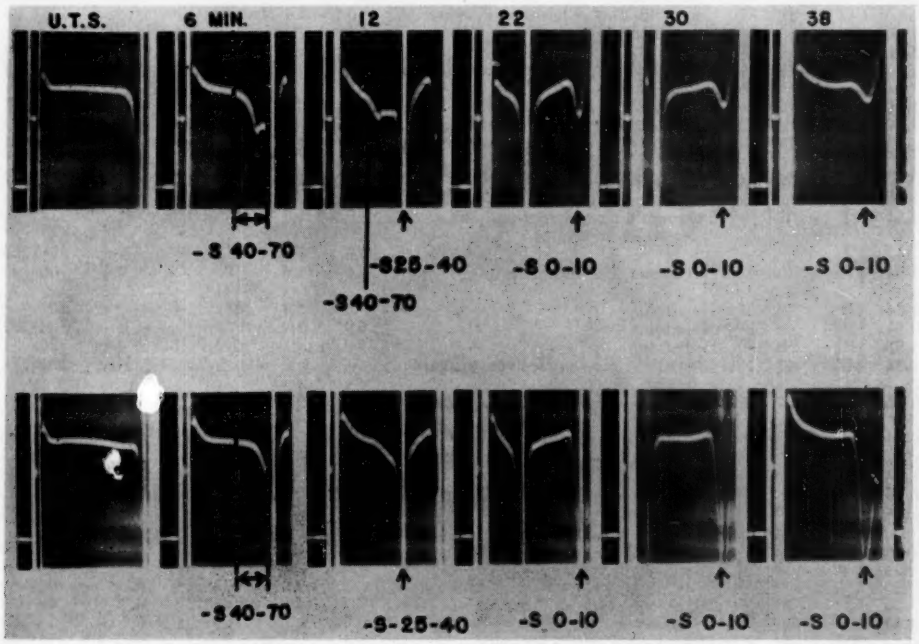


FIG. 1. PT. M.L., FEMALE, AGE 63, WITH CHOLELITHIASIS AND MINIMAL BILIARY CIRRHOSIS—REPRESENTATIVE ANALYTICAL ULTRACENTRIFUGE PATTERNS OF ICTERIC (PREOPERATIVE) AND POST-ICTERIC (POSTOPERATIVE) STAGES OF HER ILLNESS

The interval between the above analyses is approximately 3 months. Additional preoperative serum data include: total cholesterol 249 mg. per cent (esters 33 per cent), lipid phosphorus $\times 25 = 475$ mg. per cent phospholipid, van den Bergh 7 mg. per cent (total), alkaline phosphatase 11 Bodansky units per 100 ml. The post-icteric pattern was associated with the following laboratory data: total cholesterol 211 mg. per cent (esters 64 per cent), lipid phosphorus $\times 25 = 325$ mg. per cent phospholipid. In the upper (icteric) pattern the $-S_{1.21}$ 0–12 lipoprotein peak is small, there is a heavy refractive band in the $-S_{1.21}$ 25–40 ($S_f$ 0–12) region indicating a high lipoprotein concentration and a distinct $-S_{1.21}$ 40–70 ($S_f$ 12–20) component is visible. The lower patterns show a greater than 3-fold increase in the $-S_{1.21}$ 0–12 lipoprotein concentration, a reduced $-S_{1.21}$ 25–40 lipoprotein concentration and virtual disappearance of the $-S_{1.21}$ 40–70 class of lipoproteins. (Both analyses at 5 $C_e$.)

TABLE

*Lipoprotein spectrum and hepatic*

| Date | Patient | Age | Sex | Diagnosis | van den Bergh | | Alkaline phosphatase, B.U. | Thymol turbidity *units/100 cc. serum* | Cephalin flocculation (48 hours) | Total cholesterol *mg. %* | Per cent esterified | Mg. % Lipid P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total | Direct | | | | | | |
| 1953-54 & 55 | DP | 26 | F | Primary (xanthomatous) biliary cirrhosis† | 17.1 | 10.2 | 146 | 9 | 2–3+ | 550 | 21 | 50.0 |
| 1954-55 | CD | 55 | F | Primary (xanthomatous) biliary cirrhosis‡ | 19.7 | 12.5 | 49 | 6.2 | 3+ | 514 | 20 | 45.0 |
| 2-13-54 | ML | 63 | F | Early biliary cirrhosis, cholelithiasis | 10.5 | 6.3 | 11.3 | | 2+ | 249 | 33 | 19.0 |
| 5-4-54 | ML | | | | 0.67 | 0.28 | 4.6 | | | 211 | 69 | 12.7 |
| 1-19-54 | CAL | 59 | M | Biliary cirrhosis, carcinoma head of pancreas | 21.6 | | 6.4 | | | 233 | 18 | 20.4 |
| 4-5-54 | CO'D | 51 | M | Common duct obstruction, gastric carcinoma | 8.0 | 4.4 | 7.4 | | | 301 | 22 | 25.0 |
| 4-12-54 | CO'D | | | | | | 10.0 | | | 337 | 19 | 25.1 |
| 12-8-53 | OC | 74 | M | Common duct obstruction, carcinoma head of pancreas | 16.9 | 10.6 | 14.6 | 1.6 | 1+ | 228 | 18 | 20.5 |
| 4-20-54 | CK | 74 | F | Common duct obstruction, carcinoma common duct | 15.0 | | | | 3+ | 570 | 2 | 51.2 |
| 12-1-53 | RW | 78 | F | Common duct obstruction, carcinoma head of pancreas, liver | 15.3 | | 15.6 | | 2+ | 157 | | 21.0 |
| 2-3-54 | WRJ | 68 | M | Common duct obstruction, choledocholithiasis | 2.7 | 1.91 | | | Neg. | 127 | 39 | |
| 1-19-54 | CJ | 64 | F | Common duct obstruction, post operative adhesions | 2.0 | 1.0 | 13.3 | | Neg. | 233 | 56 | 16.0 |
| 4-6-54 | CJ | | | | 2.8 | 1.1 | 14.0 | | Neg. | 274 | 64 | 17.3 |
| 10-25-54 | JW | 46 | M | Hepatoma, Laennec's cirrhosis | 5.6 | 1.6 | 9.4 | 5 | 4+ | 66 | 42 | 7.9 |
| 11-29-54 | JC | 44 | M | Hepatoma | 7.68 | 5.44 | 28.2 | 8 | 2+ | 307 | | 26.5 |
| 3-10-54 | SW | 68 | F | Hepatoma | 4.9 | 1.9 | | | 4+ | 100 | 35 | 6.0 |
| 1-19-55 | HB | 58 | F | Hepatoma | 3.0 | | | 6.2 | 4+ | 333 | 11 | 28.3 |
| 2-11-54 | MB | 47 | F | Metastatic carcinoma liver, primary breast | | | | | Neg. | 504 | 15 | 44.6 |
| 1-20-55 | MV | 51 | F | Metastatic carcinoma liver, primary rectum | 13.7 | 9.5 | 8.2 | 1.3 | 2+ | 116 | 11 | 7.6 |
| 1-19-54 | NB | 38 | F | Cholecystitis, pericholangitis | 13.9 | | | | 4+ | 240 | 30 | 18.7 |
| 3-5-54 | MS | 10 | M | Infectious hepatitis | 4.11 | 4.01 | | | 3+ | 430 | 3 | 33.1 |
| 10-24-54 | MS | | | | | | | | | 134 | 68 | 9.0 |
| 7-16-55 | MS | | | | 0.32 | | | | | 122 | 75 | 4.0 |
| 3-9-54 | PM | 29 | M | Infectious hepatitis | 3.9 | | | 15 | 4+ | 185 | 42 | 7.7 |
| 3-12-54 | PM | | | | | | | | 1+ | 168 | 58 | 8.6 |
| 12-27-54 | LW | 57 | M | Homologous serum jaundice | 3.6 | 2.9 | 13.6 | 35 | | 242 | 64 | 15.6 |
| 2-8-55 | LW | | | | | | | | | 198 | 56 | 14.2 |
| 3-23-54 | LD | 35 | F | Homologous serum jaundice | 17.7 | 14.9 | 4.2 | | 4+ | 149 | 19 | 14.3 |
| 10-23-54 | LD | | | | 0.54 | | | | | 153 | 68 | 9.9 |
| 4-3-54 | EP | 22 | F | Homologous serum jaundice | 5.5 | 2.5 | 2.5 | | 4+ | 154 | 46 | 11.4 |
| 10-2-54 | EP | | | | | | | | | 225 | 73 | 11.5 |
| 7-18-55 | EP | | | | | | | | | 185 | 64 | 11.3 |
| 10-2-53 | Van | 48 | M | Laennec's cirrhosis, jaundice | 8.8 | 4.3 | | 2 | | 531 | 68 | 18.6 |
| 8-27-54 | JA | 15 | M | Unclassified: probable post-hepatitis cholangiolitic cirrhosis | 14.0 | 8.6 | 8.9 | 14.2 | 4+ | 31 | | 4.5 |
| 8-30-54 | JA | | | | | | | | | 56 | | 3.1 |
| 9-1-54 | JA | | | | | | | | | 26 | | 4.2 |
| 9-3-54 | JA | | | | 28.8 | 16.9 | 4.7 | 2.9 | | 26 | | 4.2 |
| 3-29-55 | GN | 55 | M | Unclassified cirrhosis | 1.1 | 0.6 | 1.9 | 16 | 4+ | 186 | 54 | 11.7 |
| 7-30-54 | LG | 54 | F | Unclassified cirrhosis | 14.9 | 6.5 | 6.4 | | | 134 | 62 | 9.2 |
| 8-2-54 | LG | | | | 14.2 | 6.1 | 8.3 | | 4+ | 113 | 60 | 9.8 |
| 8-18-54 | LG | | | | | | | | | 105 | | 8.4 |
| 3-2-54 | JG | 77 | M | Probable cirrhosis, unknown type | 18.9 | | 17.3 | | 4+ | 185 | 29 | 15.6 |
| 5-19-54 | GK | 77 | M | Undiagnosed disease, no jaundice | | | | | | 154 | 62 | 8.2 |
| 1-5-55 | GK | | | Undiagnosed jaundice and hepatosplenomegaly | | | | | | 61 | 28 | 6.8 |

* "Normal" values in mg. per cent for lipoproteins determined by the method used in this study are as follows (20, 36): $-S_{1.21} > 70 = 36.7 \pm 3.8$; $-S_{1.21}$ 40–70 $= 24.2 \pm 1.8$; $-S_{1.21}$ 25–40 = 250 (approximately 20 per cent of males and 9 per cent of females have values in excess of 250 mg. per cent); $-S_{1.21}$ 20–25 $= 11.6 \pm 1.8$; $-S_{1.21}$ 1–12 $= 176.9 \pm 6.5$.

I

*function in obstructive jaundice*

| Ratio: Mg. % Lipid P × 25 / Tot. cholesterol | Ratio: Mg. % Lipid P × 25 / Free cholesterol | Lipoproteins *mg. %** −S >70 | −S 40–70 | −S 25–40 | −S 20–25 | −S 0–12 | Ratio: −$S_{f}$ 0–12 / Tot. cholesterol | Ratio: −$S_{f}$ 0–12 / Free cholesterol | Total serum Protein/Globulin *gm./100 cc.* | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| 2.27 | 2.87 | | 664.8 | 704.4 | Absent | Absent | 0.00 | 0.00 | 7.8/3.9 | Liver biopsy |
| 2.19 | 2.74 | | 766 | 571.1 | Absent | Absent | 0.00 | 0.00 | 8.9/4.0 | Liver biopsy |
| 1.91 | 2.84 | | 99 | Obscured | 12 | 104 | 0.41 | 0.62 | 6.8/3.4 | Liver biopsy |
| 1.51 | 4.82 | | 32 | Obscured | 12 | 344 | 1.63 | 5.20 | 7.0/3.4 | 2½ months postoperative, no jaundice |
| 2.19 | 2.67 | 32 | 54 | Obscured | 5 | 47 | 0.20 | 0.24 | | Liver biopsy |
| 2.08 | 2.68 | | 77 | Obscured | 4.5 | 123 | 0.41 | 0.53 | | |
| 1.86 | 2.30 | | 99 | Obscured | Absent | 120 | 0.36 | 0.44 | | Laparotomy |
| 2.25 | 2.74 | | 120 | Obscured | 5 | 62 | 0.27 | 0.33 | 6.6/3.1 | −S 40–70, triple peak. Laparotomy |
| 2.25 | 2.29 | | 29 | Obscured | Absent | 72 | 0.13 | 0.13 | | Laparotomy |
| 3.34 | | | 30 | Obscured | 20 | 16 | 0.10 | | | Laparotomy |
| | | | 50 | 187 | Absent | 86 | 0.68 | 1.12 | 7.2/2.6 | Laparotomy |
| 1.72 | 3.88 | | 32 | Obscured | 14 | 239 | 1.02 | 2.32 | | Laparotomy |
| 1.58 | 4.46 | | 35 | Obscured | 9 | 492 | 1.80 | 5.07 | | |
| 3.00 | 5.21 | | 31 | 151 | 3.6 | 7 | 0.11 | 0.19 | 6.55/3.54 | Autopsy. Ceph. floc. 4+ at 24 hours |
| 2.16 | | | 120 | 254 | 7 | 29 | 0.09 | | 6.7/2.6 | Autopsy |
| 1.50 | 2.31 | | 23 | Obscured | 12 | 9 | 0.09 | 0.14 | 3.7/1.6 | Autopsy |
| 2.13 | 2.39 | | 86 | 542 | Absent | 72 | 0.22 | 0.24 | 7/? | Liver biopsy |
| 2.21 | 2.60 | | 133 | Obscured | 17 | 16 | 0.027 | 0.036 | | Liver biopsy, clinically jaundiced |
| 1.64 | 1.84 | 92 | 81 | 139 | Absent | 158 | 1.36 | 1.53 | 7.0/2.9 | Laparotomy |
| 1.95 | 2.80 | | 54 | Obscured | 3.6 | 43 | 0.18 | 0.25 | | Liver biopsy |
| 1.93 | 1.98 | 76 | 94 | Obscured | 38 | 110 | 0.25 | 0.26 | 6.5/? | |
| 1.68 | 5.36 | | 1.8 | 117 | 14 | 250 | 1.87 | 5.91 | | Clinically well, asymptomatic |
| 1.64 | 6.45 | | 14 | 102 | 21 | 222 | 1.82 | 7.16 | | Clinically well, asymptomatic |
| 1.04 | 1.79 | 51 | 53 | Obscured | 9 | 85 | 0.46 | 0.78 | 6.2/2.3 | Cephalin 4+ in 24 hours. Clinically jaundiced |
| 1.28 | 3.12 | 146 | 52 | Obscured | 5 | 84 | 0.50 | 1.22 | | |
| 1.61 | 4.53 | 113 | 74 | 284 | 1.8 | 105 | 0.43 | 1.22 | | |
| 1.79 | 4.08 | 61 | 50 | 320 | 14 | 279 | 1.41 | 3.23 | | Liver function tests all normal |
| 2.40 | 2.93 | | 94 | Obscured | Absent | 102 | 0.68 | 0.83 | 7.8/3.9 | Cephalin 4+ at 24 hours |
| 1.62 | 5.77 | | 42 | 151 | 14 | 197 | 1.29 | 4.00 | | Asymptomatic |
| 1.85 | 3.43 | 66 | 55 | Obscured | 15 | 90 | 0.59 | 1.08 | | Cephalin 4+ at 24 hours |
| 1.28 | 4.64 | 86 | 52 | 291 | 12 | 161 | 0.72 | 2.61 | | van den Bergh normal 5-7-54 |
| 1.53 | 4.29 | | 105 | 198 | 12 | 165 | 0.89 | 2.48 | | Asymptomatic |
| 0.88 | 2.77 | 93 | 72 | Obscured | 8.5 | 79 | 0.15 | 0.48 | 5.4/3.0 | Still jaundiced 2 years later |
| 3.65 | | | 9 | 102 | 1.8 | 5 | 0.18 | | 8.7/6.1 | Liver biopsy |
| 1.39 | | | 14 | 87 | 1.8 | 11 | 0.20 | | | |
| 4.04 | | | 13 | 90 | 5 | 9 | 0.36 | | | |
| 4.04 | | | 16 | 86 | 4.5 | 16 | 0.63 | | 7.1/4.8 | |
| 1.58 | 3.41 | 72 | 58 | 267 | 3.6 | 182 | 0.98 | 2.11 | 7.4/4.6 | Liver biopsy |
| 1.72 | 4.51 | | 36 | 159 | 4.5 | 169 | 1.27 | 3.31 | 8.0/4.3 | |
| 2.17 | 5.33 | | 25 | 157 | 8 | 142 | 1.25 | 3.10 | | |
| 2.00 | | | 23 | 161 | 10 | 64 | 0.60 | | | |
| 2.05 | 2.88 | | 80 | Obscured | Absent | 41 | 0.23 | 0.32 | 6.5/3.2 | Liver biopsy not diagnostic |
| 1.33 | 3.48 | | 32 | Obscured | 3.6 | 121 | 0.78 | 2.03 | | |
| 2.79 | 3.86 | | 41 | 128 | Absent | 73 | 1.21 | 1.67 | | Deeply jaundiced, deceased 1-7-55 |

† Values reported are means of serial observations made over 2-year period.
‡ Values reported are means of serial observations made over 6-month period.

protein:total cholesterol ratio was determined in all 28 patients and in 23 it fell below 0.73. One of the five patients whose ratios exceeded this value had a value of only 0.78 (GK). The $-S_{1.21}$ 0–12 lipoprotein:free cholesterol ratio, determined in 25 of these patients, fell below 2.63 in 24 cases. One or both ratios were abnormally low in 27 of the 28 patients in the study. As a rule the lower values of these ratios were seen in the more deeply jaundiced patients, but there were exceptions (*e.g.*, LG).

When jaundice subsides following relief of obstruction, high density $-S_{1.21}$ 0–12 lipoprotein levels increase (Figure 1), serum free cholesterol levels return toward normal (if previously abnormal) resulting in an increase in the per cent of cholesterol esterified, and the ratios $-S_{1.21}$ 0–12 lipoprotein:total cholesterol and $-S_{1.21}$ 0–12 lipoprotein:free cholesterol increase to or above values for the normal adult male. As improvement continues, elevated concentrations of low density $-S_{1.21}$ 25–40 and 40–70 lipoproteins fall toward normal, resulting in increased $-S_{1.21}$ (0–12/25–70) lipoprotein ratios. If progressive disease is present, further decrease in the value of these ratios is usually noted.

The data indicate that most of the serum cholesterol and phospholipids, even when markedly increased in amount, are present as low density $-S_{1.21}$ 25–40 ($S_f$ 0–12) lipoproteins, and to a lesser degree, as lower density lipoproteins of $-S_{1.21}$ 40–70 ($S_f$ 12–20) class. Inhomogeneity of the low density group of lipoproteins was occasionally evidenced by the appearance of multiple peaks in the $-S_{1.21}$ 40–70 region of the analytical patterns (Figure 2).

The presence of low density lipoproteins of flotation rates in excess of $-S_{1.21} > 70$ ($S_f > 20$) was noted in only eight subjects, and no correlation was apparent between these low density lipoproteins and other laboratory or clinical data, with the possible exception of hepatitis, since five of six subjects with either homologous serum jaundice or infectious hepatitis exhibited relatively small amounts of these lipoproteins.

It is of interest to note that the value of the ratio phospholipid:free cholesterol was less than 2.0 in only three subjects (MV, metastatic carcinoma, 1.84; MS, and PM, each with infectious hepatitis, 1.98 and 1.79, respectively). This study amply confirms the observations of Jackson and his co-workers (1) mentioned previously. In this regard it is of further interest to note that phospholipid:free cholesterol ratios in native serum as well as in Cohn fractions IV + V + VI, and I + III in a healthy woman and in a woman with primary biliary cirrhosis, calculated from the data of Table I in the paper by Russ, Raymunt, and Barr (10), do not exceed the value 2.0 in any instance.

There is a positive correlation between the serum albumin and total cholesterol levels ($r = 0.585$, significant at the 0.02 level) in the 15 subjects in whom a total of 17 albumin levels was determined.

## DISCUSSION

The mechanism or mechanisms responsible for the increment in serum free cholesterol and phos-

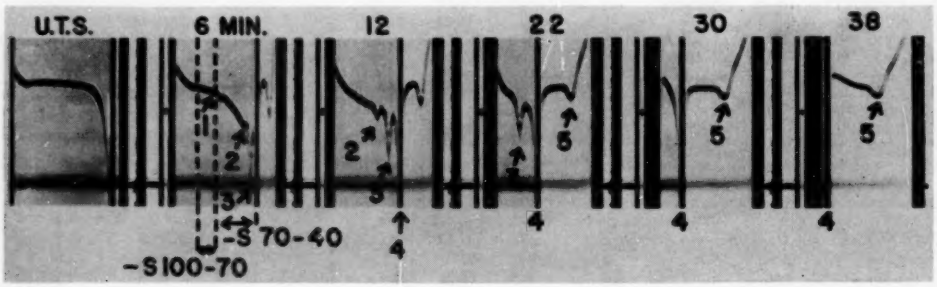


FIG. 2. PATIENT O.C., MALE, AGE 74, WITH COMMON DUCT OBSTRUCTION DUE TO CARCINOMA OF THE HEAD OF THE PANCREAS—ANALYTICAL ULTRACENTRIFUGE PATTERN AT 5 $C_0$ SHOWING THREE "SUB-SPECIES" OF LOW DENSITY LIPOPROTEINS UNDERGOING FLOTATION WITHIN THE LIMITS $-S_{1.21}$ 40–70 (NO.'S 1, 2, AND 3)

The principal lipoprotein peak (No. 4) is obscured by a refractive band due to the high concentration present. The No. 5 peak is a high density lipoprotein here undergoing flotation at an average rate of approximately $-S_{1.21}$ 12.

pholipid levels, and for the failure of the esterified cholesterol fraction to increase, are not understood. Simple retention of bile, increased plasma cholate (20), alteration in serum cholesterol esterifying enzyme activity (21, 22) and increased hepatic cholesterol synthesis (23) have been suggested in explanation. While as much as 50 per cent of the cholesterol absorbed from the intestinal tract is present as cholesterol ester in the thoracic duct lymph (24), animal studies by Friedman and Byers (25) indicate that the liver is importantly concerned with the supply as well as withdrawal of plasma ester cholesterol. Inability of the liver to remove cholesterol from the blood for esterification may be an important reason for the relatively or absolutely low levels of plasma ester cholesterol commonly seen in biliary obstruction. Inspection of the table indicates that in only one instance (Van) out of 37 determinations did the absolute level of esterified cholesterol exceed the upper range of normal for this fraction (200 mg. per cent), while 15 determinations were below the lower range of normal (70 mg. per cent). The remaining values were within commonly accepted normal limits by the methods employed for their determinations.

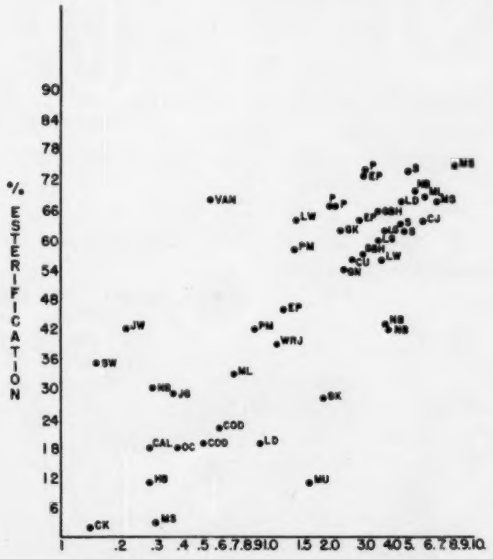


FIG. 3. SEMILOGARITHMIC PLOT OF PER CENT CHOLESTEROL ESTER (ORDINATE) AS A FUNCTION OF THE RATIO, $-S_{1.21}$ 0–20 LIPOPROTEIN : UNESTERIFIED CHOLESTEROL (ABSCISSA), IN 26 SUBJECTS DURING ICTERIC AND POST-ICTERIC PHASES OF HEPATOBILIARY DISEASE

The coefficient of correlation (r) between these two functions is 0.77 ($p < 0.001$), z (35) value $1.02 \pm 0.155$. The regression equations are: $y = 33.9 \times \pm 41.8$ and $x = 0.018\ y - 0.683$.

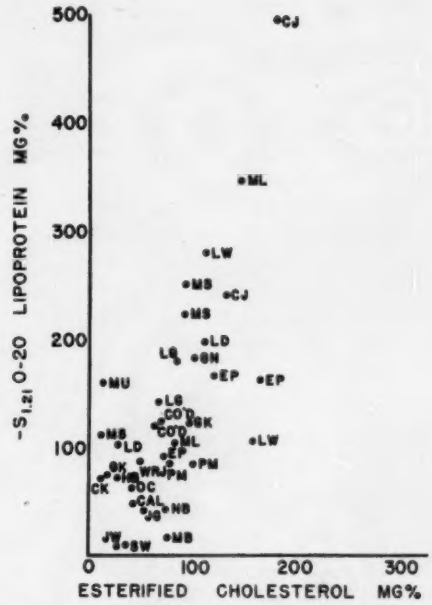


FIG. 4. RECTILINEAR PLOT OF ESTERIFIED CHOLESTEROL CONCENTRATION (ABSCISSA) AS A FUNCTION OF THE $-S_{1.21}$ 0–20 LIPOPROTEIN CONCENTRATION (ORDINATE)

The coefficient of correlation (r) between these two functions is 0.68, ($p < 0.001$) z value $0.82 \pm 0.18$. The regression equations are $y = 0.3x + 36.6$ and $x = 1.5\ y + 16.8$.

Because reduced $-S_{1.21}$ 0–12 lipoprotein concentrations and $-S_{1.21}$ 0–12 lipoprotein:free cholesterol ratios, as well as lower esterified cholesterol fractions, are common to obstructive jaundice, these data were examined for possible correlations. In Figure 3 the value of the ratio $-S_{1.21}$ 0–12 lipoprotein:free cholesterol is plotted semilogarithmically as a function of the per cent esterification of the serum cholesterol in 26 patients in whom these data are available from 45 determinations. In Figure 4 the concentration of esterified cholesterol is plotted on rectilinear coordinates as a function of the $-S_{1.21}$ 0–12 lipoprotein concentration in these same subjects. Both plots show a highly significant positive correlation. It is possible therefore to predict the $-S_{1.21}$ 0–12 lipoprotein concentration in patients

with obstructive jaundice from knowledge of either the concentration of the cholesterol esters, or the total or free cholesterol concentration, together with the per cent of cholesterol esterified.

The metabolic significance of these correlations is not evident from this study. Several possibilities suggest themselves. The factors responsible for the relative inability of the liver to esterify cholesterol in obstructive jaundice, or for the reduction in cholesterol-esterifying enzyme activity, may retard or otherwise inhibit the synthesis of high density lipoproteins. Alternatively, these factors may modify the lipid composition of these lipoproteins, reducing their density.

From the serum of healthy subjects Cohn fractions IV + V + VI yield lipoproteins which appear to be similar to if not identical with the high density $-S_{1.21}$ 0–12 lipoproteins. It is clear that this similarity no longer exists in obstructive jaundice, since in this disorder the lipid recovery from Cohn fractions IV + V + VI is increased (2, 3, 9, 10). It is possible that high density $-S_{1.21}$ 0–12 lipoproteins acquire additional lipid in obstructive jaundice, thereby rendering them "low density" lipoproteins, but retain solubility characteristics that determine their presence in the Cohn fractions IV + V + VI. Other differences have been suggested by Russ, Raymunt, and Barr (10). Reported analyses of the high density ($> 1.063$) lipoproteins found in the serum of patients with biliary obstruction are scant (9, 10) and do not take cognizance of the relatively large "lipid" phosphorus fraction (5 to 10 per cent of the "lipid" phosphorus concentration of native serum) found in the 1.063 and 1.21 "infranates" which is insoluble in petroleum ether (9 addendum, 15). Further studies of these lipoproteins are necessary.

The observations we have been able to make in two patients with metastatic carcinoma of the liver (MB and MV) are of interest in view of the reports by other workers (26, 27) in which low "alpha" lipoprotein levels were noted in advanced carcinoma of the breast, especially when bone metastases were present. It is possible that the reduced "alpha" lipoprotein concentrations in these patients are due in significant measure to liver metastases which lead to some degree of biliary obstruction. The lowest values for the ratios $-S_{1.21}$ 0–12 lipoprotein:total cholesterol and $-S_{1.21}$ 0–12 lipoprotein:free cholesterol in the studies reported here were noted in the subject with carcinoma of the breast with metastases (MB). High density $-S_{1.21}$ 0–12 lipoproteins are readily diminished by androgenic and increased by estrogenic steroids (28). Studies of the serum lipoproteins in patients with breast cancer, therefore, must take cognizance of any recent therapy with gonadal steroids or related compounds.

It is likely that a variety of types of liver damage may reduce the ability of the liver to synthesize high density lipoproteins. Total body radiation studies described by Entenman, Neve, and Olmstead (29) and by others (30) suggest the possibility that liver damage may result from such radiation and be reflected in the plasma by a reduced level of high density lipoproteins. In view of the increase in high density lipoprotein concentration caused by estrogen administration, it is interesting to speculate on the mechanism of the protection against acute radiation mortality that estrogens afford (31–34).

## SUMMARY AND CONCLUSIONS

Serum lipid and lipoprotein determinations were made in 26 subjects with obstructive jaundice of varying etiology. Ultracentrifugation employing a solvent density of 1.21 g. per ml. was utilized for lipoprotein analysis.

The following conclusions were derived:

1. Obstructive jaundice, regardless of type, is characterized by: (a) reduced or absent high-density ($> 1.063$) lipoproteins of flotation rate $-S_{1.21}$ 0–20; (b) increased $-S_{1.21}$ 25–40 and $-S_{1.21}$ 40–70 lipoproteins.

2. Low density lipoproteins of the flotation rate class $-S_{1.21}$ 70–100 may be present in the serum from patients with homologous serum hepatitis, but as a rule are absent in other types of obstructive jaundice.

3. There exists a highly significant and positive correlation between the per cent esterification of serum cholesterol and the logarithm of the ratio $-S_{1.21}$ 0–20 lipoprotein:free cholesterol. (This ratio was selected to provide an index of high density lipoprotein levels in relation to the serum cholesterol concentrations.)

4. There exists a highly significant and positive correlation between the concentrations of esterified cholesterol and $-S_{1.21}$ 0–20 lipoproteins.

5. The concentration of high density lipoproteins in obstructive jaundice may be predicted from knowledge of either the concentration of the cholesterol esters or the total or unesterified cholesterol concentration together with the per cent of cholesterol esterified.

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# NET POTASSIUM MOVEMENT BETWEEN RESTING MUSCLE AND PLASMA IN MAN IN THE BASAL STATE AND DURING THE NIGHT[1]

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Net movement of potassium between cells and extracellular fluid has been demonstrated in a variety of experimental conditions by direct analysis of muscle, by metabolic balance techniques, and by measurement of differences in potassium concentration in arterial blood and venous blood draining muscle. It has, for example, been demonstated that potassium leaves muscle during exercise, anoxia, potassium depletion, and under the influence of acidosis and of adrenal cortical hormones (1).

The present study demonstrates that there are, in addition, diurnal fluctuations in movement of potassium between skeletal muscle and extracellular fluid. Net movement of potassium between plasma and muscles of the forearm has been determined in human subjects by a technique which permits continuous monitoring of movement of potassium in relatively undisturbed muscle. Concentration of potassium is measured in arterial plasma and in venous plasma draining the deep forearm tissues. Plasma flow through the forearm is measured by the dye-dilution method (2). The product of flow, F, and the difference in arteriovenous concentration, A-V, defines $\dot{Q}$, the net quantity of potassium entering or leaving the forearm tissues per unit time.

## METHODS

*In vivo studies.* Twenty-one male subjects (16 medical students and laboratory personnel and five convalescent ambulatory patients) were studied in the basal state between the hours of 10 A.M. and 1 P.M. Three medical students, including two of the 21 subjects above, were also studied throughout the night and morning hours; *i.e.*, from 10 P.M. to 11 A.M. Studies of carbohydrate metabolism in the first 13 of these subjects have been reported elsewhere (3, 4) and subject numbers in those papers correspond to those reported here. All subjects had their last meals between 6 and 7 P.M. and were at rest for at least one hour before blood was sampled. The arm was supported comfortably, and there was no overt activity of the forearm muscles during the period of study. Blood from the hand was eliminated by inflating to above systolic pressure a cuff about the wrist, applied five minutes prior to blood sampling. Blood was collected in heparinized syringes; metabolic inhibitors were not used. Consecutive pairs of arteriovenous samples were obtained at 12 to 60 minute intervals. Processing of blood was as expeditious as possible. Immediate centrifugation of the blood samples was carried out for ten minutes, plasma was transferred and re-centrifuged twice. A few visibly hemolyzed samples were discarded. Since 0.05 per cent hemolysis can be detected visually, the maximum increase in plasma potassium concentration resulting from undetected hemolysis was 0.02 mEq. per L. Handling of samples for potassium analysis was carried out at room temperature (25° C) to minimize potassium and water shifts in erythrocytes (5) prior to their separation from plasma.

*In vitro studies.* A sample of antecubital venous blood was obtained anaerobically in a heparinized syringe. A drop of mercury was introduced and the sample was thoroughly mixed. Samples of blood were transferred anaerobically into duplicate syringes. Into one of these, 100 per cent $O_2$ was introduced, and into the other a mixture of 5.0 to 6.4 per cent $CO_2$ in $N_2$. Gas volumes were varied to obtain the desired range of gas concentrations in the blood samples. Syringes were capped and the blood and gas phases were equilibrated at room temperature by rotation for ten minutes. Gases were expelled and blood was then handled as in the *in vivo* studies. Hematocrit determinations (Wintrobe) were performed in duplicate. Tubes were centrifuged for one hour at 1500 g. and no correction was made for trapped plasma. In some studies, concentrations of $O_2$ and $CO_2$ were determined by the method of Van Slyke and Neill (6).

*Potassium analysis.* Duplicate or triplicate dilutions of plasma were made and concentrations were measured

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with the Beckman Model DU flame photometer. The precision of the analysis is as follows: the standard error of the estimate of the mean concentration from duplicate analyses of a single blood sample is 0.04 mEq. per L. The standard error of the estimate of a single arteriovenous difference then is 0.06 mEq. per L. for samples diluted in duplicate. On the average, three pairs of arterial and venous samples were obtained from each subject in the basal state and only the mean of these multiple samples is reported. In general, the standard error (analytical) of the estimate of *mean* A-V difference to be presented for each subject in Table I is 0.03 mEq. per L. For the entire series of 21 subjects, the standard error of the estimated overall mean A-V difference is less than 0.01 mEq. per L.

## RESULTS

### *Potassium movement in the basal state*

Sixty-five individual A-V differences were obtained in 21 subjects studied 16 to 19 hours postprandially. In only seven samples was the arterial concentration higher than the venous. The mean A-V difference in 19 of the 21 subjects was negative, the grand mean being − 0.23 mEq. per L. (Table I). The net loss of potassium from tissue to blood in 15 subjects averaged 0.66 μEq. per min. per 100 ml. forearm tissue. $\dot{Q}$ can be calculated in terms of forearm *muscle* by multiplying 0.66 by 4/3 (see Reference 3), giving a mean loss of 0.88 μEq. per min. per 100 g. forearm muscle.

TABLE I

*Movement of potassium in the basal state* *

| Subject | Number of pairs | $A_K$ mEq./L. | $(A\text{-}V)_K$ mEq./L. | $\dot{Q}_K$ μEq./min./100 ml. forearm |
|---|---|---|---|---|
| 1 | 6 | 4.36 | −0.31 | −0.88 |
| 2 | 6 | 4.19 | −0.46 | |
| 3 | 6 | 4.07 | −0.15 | |
| 4 | 3 | 3.90 | −0.23 | −0.50 |
| 5 | 3 | 4.28 | −0.01 | |
| 6 | 3 | 3.90 | −0.23 | −0.37 |
| 7 | 2 | 3.77 | −0.16 | |
| 8 | 1 | 3.82 | −0.14 | −0.17 |
| 9 | 1 | 3.72 | −0.03 | −0.10 |
| 10 | 1 | 4.49 | −0.38 | −0.83 |
| 11 | 3 | 4.51 | −0.10 | |
| 12 (C) | 2 | 3.91 | −0.07 | −0.18 |
| 13 (K) | 3 | 4.04 | −0.09 | −0.17 |
| 14 | 3 | 4.14 | −0.96 | −2.19 |
| 15 | 3 | 3.89 | −0.44 | −1.86 |
| 16 | 3 | 4.03 | +0.01 | +0.11 |
| 17 | 3 | 3.98 | −0.64 | −2.69 |
| 18 | 4 | 3.94 | −0.28 | |
| 19 | 3 | 3.65 | +0.23 | +0.54 |
| 20 | 3 | 4.04 | −0.16 | −0.21 |
| 21 | 3 | 3.95 | −0.19 | −0.43 |
| Mean | 3 | 4.03 | −0.229 | −0.662 |
| S.D. | | 0.233 | 0.253 | 0.902 |
| S.E.M. | | 0.051 | 0.055 | 0.233 |

* $A_K$ is the concentration of potassium in arterial plasma. $(A-V)_K$ is the arteriovenous difference in plasma concentration of potassium. $\dot{Q}_K$ is the calculated net potassium movement; the minus sign indicates that net movement was from muscle to plasma. Values in each column are the means for each subject. Plasma flow was not successfully measured in six subjects (see Reference 2), and $\dot{Q}_K$ for each of these was therefore not calculated. S.D. is the standard deviation and S.E.M. is the standard error of the mean.

TABLE II

*Movement of potassium during the night* *

| Sample | Time | $A_K$ mEq./L. | $(A\text{-}V)_K$ mEq./L. | $\dot{Q}_K$ μEq./min./100 ml. forearm |
|---|---|---|---|---|
| Subject C: | | | | |
| 1 | 11:43 P.M. | 3.91 | −0.32 | −0.89 |
| 2 | 1:00 A.M. | 3.91 | +0.03 | +0.08 |
| 3 | 2:00 | 4.17 | +0.35 | +1.12 |
| 4 | 3:00 | 3.86 | −0.09 | −0.25 |
| 5 | 5:00 | 3.81 | −0.09 | −0.40 |
| 6 | 7:00 | 3.88 | −0.04 | −0.07 |
| 7 | 9:00 | 3.84 | −0.17 | −0.42 |
| 8 | 10:00 | 3.90 | −0.03 | −0.13 |
| 9 | 11:00 | 3.92 | −0.11 | −0.23 |
| Subject K: | | | | |
| 1 | 11:53 P.M. | 3.93 | −0.12 | −0.23 |
| 2 | 1:00 A.M. | 3.95 | | |
| 3 | 2:00 | 3.94 | +0.01 | +0.02 |
| 4 | 3:30 | 3.92 | −0.02 | −0.04 |
| 5 | 5:00 | 3.89 | −0.11 | −0.17 |
| 6 | 6:30 | 4.16 | −0.06 | −0.07 |
| 7 | 8:00 | 4.17 | +0.02 | +0.04 |
| 8 | 10:13 | 4.03 | −0.09 | −0.18 |
| 9 | 10:35 | 4.00 | −0.09 | −0.17 |
| 10 | 11:02 | 4.10 | −0.09 | −0.17 |
| Subject N: | | | | |
| 1 | 10:00 P.M. | 3.86 | −0.31 | |
| 2 | 11:30 | 3.84 | +0.10 | |
| 3 | 1:00 A.M. | 3.76 | +0.04 | |
| 4 | 3:00 | 3.82 | +0.05 | |
| 5 | 5:00 | 3.90 | +0.04 | |
| 6 | 7:00 | 3.86 | +0.02 | |
| 7 | 8:00 | 3.82 | +0.01 | |
| 8 | 9:00 | 3.90 | +0.12 | |

* See legend to Table I for explanation of symbols.

### *Potassium movement during the night*

Results on the three subjects are given in Table II and Figure 1. Measurement of plasma flow was made in subjects C and K but was unsuccessful in subject N (2). It appears that between the hours of 1 and 8 A.M. there is no net movement of potassium, the overall mean and median A-V difference during this time being + 0.01 mEq. per

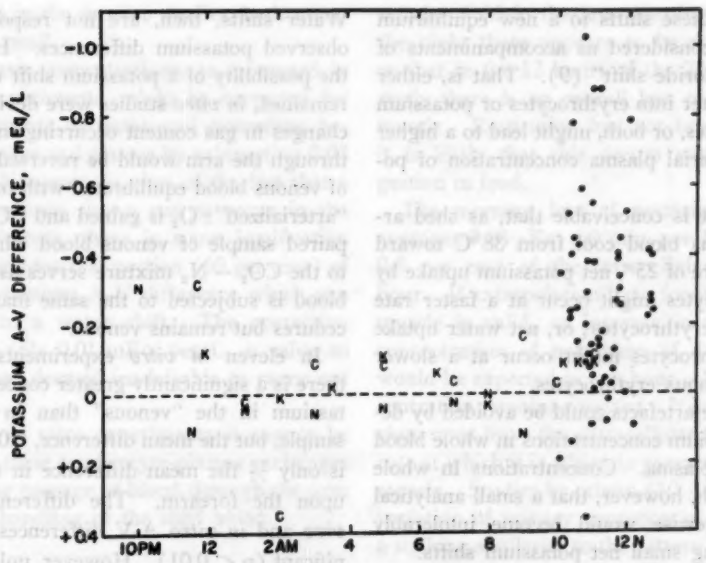


FIG. 1. TIME COURSE OF ARTERIOVENOUS DIFFERENCE IN PLASMA POTASSIUM CONCENTRATION

C, K and N refer to subjects followed during the night. Black dots represent all A-V differences obtained in nineteen additional subjects studied only between 10 A.M. and 1 P.M.

L., and only one of the 15 A-V differences lies outside the range expected from analytical error alone. These results are in contrast to the larger series of results obtained during the late morning hours. In the few observations made prior to 1 A.M. there seems also to be real loss of potassium from the forearm.

In summary, the general trend is one of potassium loss from muscle prior to 1 A.M., little or no net movement during most of the night, then increasing potassium loss in the later morning hours.

*Evidence that the observed A-V differences in potassium concentration represent net movement of potassium between forearm muscle and blood*

1) Transiently negative A-V differences might be observed if the concentration of potassium in arterial plasma were falling during the period of study with consequent net diffusion of potassium from interstitial fluid into capillaries. However, only very minor fluctuations in concentration of arterial potassium occurred and there was no consistent directional trend. On the average the concentration decreased 0.004 mEq. per L. ± 0.018 (S.E.M.) in consecutive samples.

2) Since potassium escapes from anoxic muscle (*7*), it was necessary to demonstrate that no leak of blood from ischemic hand tissues distal to the pressure cuff at the wrist occurred. Evidence that there was no such leak has been previously presented (3).

3) The chief source of the potassium added to venous blood might be forearm tissues other than muscle, that is, skin, tendon or bone. The placement of the venous catheter deeply into the forearm muscle mass minimizes possible contribution from skin. Of the potassium in the remaining tissues, there is probably 20 times as much in muscle as there is in bone and tendon combined, if concentrations in human tissues are similar to those in the rat (8). Nevertheless, a portion of the observed potassium loss may be from tissues other than muscle.

4) *In vitro* studies of net potassium and water movement between blood cells and plasma were made to examine the possibility of significant shift of potassium, of water, or of both, either (a) in the arm as arterial blood becomes venous, or (b) in shed blood during the several minutes which elapse before cells are separated from plasma.

In case (a), these shifts to a new equilibrium state might be considered as accompaniments of the classical "chloride shift" (9). That is, either movement of water into erythrocytes or potassium out of erythrocytes, or both, might lead to a higher venous than arterial plasma concentration of potassium.

In case (b) it is conceivable that, as shed arterial and venous blood cool from 38°C toward room temperature of 25°, net potassium uptake by arterial erythrocytes might occur at a faster rate than by venous erythrocytes; or, net water uptake by arterial erythrocytes might occur at a slower rate than by venous erythrocytes.

These possible artefacts could be avoided by determining potassium concentrations in whole blood rather than in plasma. Concentrations in whole blood are so high, however, that a small analytical error percentagewise would become intolerably large in detecting small net potassium shifts.

In both cases (a) and (b), in order for water shifts to account for the observed differences in potassium concentration, the volume of packed cells in venous blood would have to exceed that in arterial blood by 2.3 ml. per 100 ml. blood on the average. In 35 pairs of arterial and venous blood samples obtained in 12 of the subjects, there was no significant A-V difference, the mean being only + 0.2 ml. per 100 ml. ± 0.2 (S.E.M.). Water shifts, then, are not responsible for the observed potassium differences. However, since the possibility of a potassium shift in erythrocytes remained, *in vitro* studies were devised so that the changes in gas content occurring in blood flowing through the arm would be reversed. The sample of venous blood equilibrated with oxygen becomes "arterialized": $O_2$ is gained and $CO_2$ is lost. The paired sample of venous blood which is exposed to the $CO_2 - N_2$ mixture serves as a control: the blood is subjected to the same manipulative procedures but remains venous.

In eleven *in vitro* experiments (Table III) there is a significantly greater concentration of potassium in the "venous" than in the "arterial" sample, but the mean difference, 0.03 mEq. per L., is only 1/7 the mean difference in the experiment upon the forearm. The difference between *in vivo* and *in vitro* A-V differences is highly significant ($p < 0.01$). However, unlike the forearm experiments, a significant difference in "arterial" and "venous" hematocrits occurred in the *in vitro* studies. In only one of ten pairs was this water shift large enough to account completely for the observed difference in potassium concentration (Table III, columns headed "% Water shift" and "% Change in potassium"). There therefore appears to be not only a water shift but also a direct

TABLE III

*Movement of potassium between red cells and plasma with changes in $O_2$ and $CO_2$ contents* *

| Expt. | $A_K$ | $(A\text{-}V)_K$ | $(A\text{-}V)_{O_2}$ | $(A\text{-}V)_{CO_2}$ | $(A\text{-}V)_{Hct}$ | % Water shift | % Change in potassium |
|---|---|---|---|---|---|---|---|
| A | 3.67 | −0.01 | 9.4 | −1.6 | | | −0.2 |
| B | 3.81 | −0.13 | 13.9 | −1.6 | −0.4 | −0.7 | −3.4 |
| C | 3.95 | −0.01 | 15.4 | −3.4 | −1.4 | −2.6 | −0.4 |
| D | 3.68 | +0.01 | | | −0.1 | −0.2 | +0.3 |
| E | 3.74 | −0.03 | 13.4 | −2.3 | −0.2 | −0.4 | −0.7 |
| F | 3.73 | −0.05 | | | −0.3 | −0.6 | −1.4 |
| G | 3.91 | 0 | | | +0.1 | +0.3 | 0 |
| H | 4.19 | −0.04 | | | 0 | 0 | −1.0 |
| I | 3.61 | −0.04 | | | −0.2 | −0.3 | −1.3 |
| J | 4.05 | −0.01 | 10.8 | −5.6 | 0 | 0 | 0 |
| K | 4.01 | −0.07 | 11.6 | −6.3 | −0.7 | −1.2 | −1.7 |
| Mean | 3.850 | −0.034 | 12.4 | −3.5 | −0.31 | −0.57 | −0.96† |
| S.E.M. | | 0.0120 | | | 0.140 | 0.260 | 0.338 |

* A refers to the concentration in the "arterialized" sample; V, to that in the venous sample; A-V, to the arteriovenous difference. Potassium concentration is in mEq. per L. plasma; gas concentrations are in ml. per 100 ml. blood. Hematocrit, Hct, is in ml. packed cells per 100 ml. blood. % Water shift $= \frac{(V\text{-}A)_{plasmacrit}}{A_{plasmacrit}} 100$. % Change in potassium $= \frac{(A\text{-}V)_K}{A_K} 100$.

† Experiment A omitted from calculation of the mean since per cent water shift was not measured in this subject.

potassium shift in the *in vitro* studies, both quantitatively very small.

Venous plasma concentrations, as measured in the forearm experiments, might be corrected for possible movements of water and potassium between erythrocytes and plasma by subtracting 0.03 mEq. per L. However, in view of the fact that a water shift has been shown not to occur in the *in vivo* experiments, there is some justification for not correcting for that portion (60 per cent) of the *in vitro* potassium A-V difference which can be attributed to a water shift. The correction then would be only 0.01 mEq. per L., a value so small that it was deemed inadvisable to carry out any correction.

From these *in vitro* experiments it cannot be stated whether these erythrocyte-plasma exchanges occurred during passage of blood through the arm or during the handling of the shed blood.

## DISCUSSION

These studies demonstrate that, in addition to factors described previously as influencing transcellular movement of potassium, there is a net loss of potassium from skeletal muscle to extracellular fluid in the late morning hours. During the night there appears to be no net movement, so that in the 12 hours of the 24 included in this study there is an overall loss of potassium from muscle. Restitution has not been demonstrated; it is likely that this occurs with potassium ingestion in food.

The morning loss of potassium from muscle averages 0.88 μEq. per min. per 100 g. muscle, or 0.5 per cent of the intracellular potassium per hour. If extrapolation from forearm to total body muscle is valid, an increase in the extracellular concentration of potassium of 1.0 mEq. per L. would be expected in one hour. Yet, arterial concentration remains constant. It is clear that potassium must leave the extracellular fluid at about the rate at which it is added to extracellular fluid from muscle. Studies by others (10, 11) of the diurnal variation of urinary excretion of potassium show a striking similarity to the pattern of net potassium movement from muscle. That is, excretion falls to minimal levels during the night, and rises sharply to a maximum output at 10 or 11 A.M. (compare Figure 1). It would appear that urinary loss is the likely fate of the bulk of the potassium added to extracellular fluid from muscle;

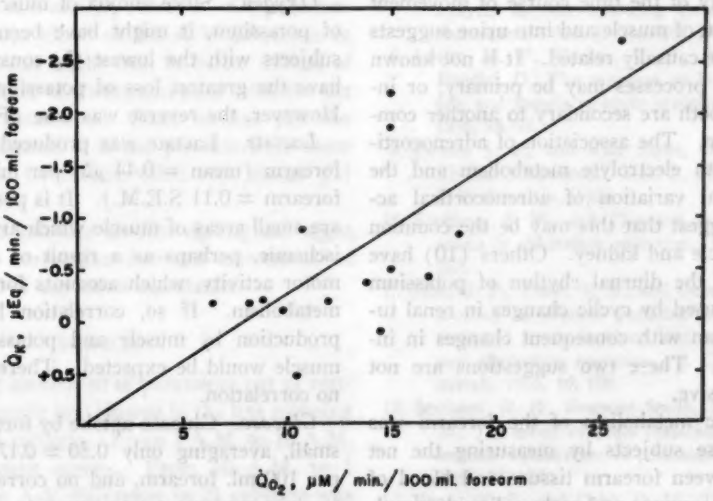


FIG. 2. CORRELATION BETWEEN OXYGEN CONSUMPTION OF FOREARM TISSUES AND MOVEMENT OF POTASSIUM BETWEEN FOREARM TISSUES AND BLOOD

Each dot represents the mean value for each subject studied in the basal state. Line of regression calculated by "least squares" method. Probability of correlation by chance, $< 0.01$.

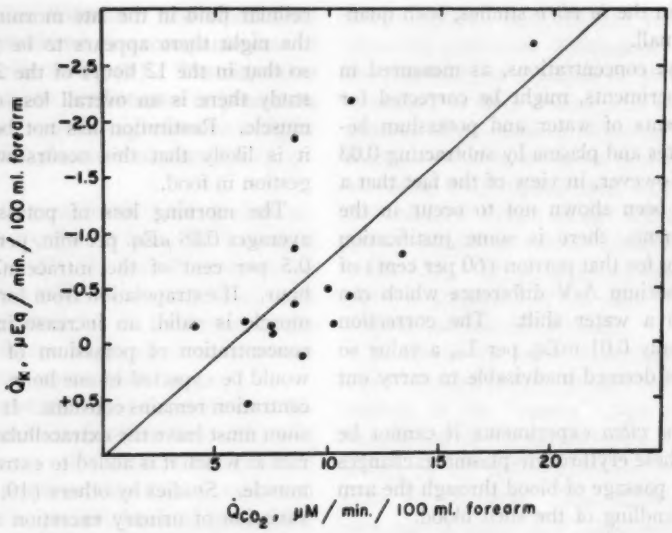


FIG. 3. CORRELATION BETWEEN $CO_2$ PRODUCTION BY FOREARM TISSUES AND MOVEMENT OF POTASSIUM BETWEEN FOREARM TISSUES AND BLOOD

Each dot represents the mean value for each subject studied in the basal state. Line of regression calculated by "least squares" method. Probability of correlation by chance, $< 0.01$.

some potassium may, however, enter the cells of other organs, for example, liver.

The similarity of the time course of movement of potassium out of muscle and into urine suggests that the two are causally related. It is not known which of these processes may be primary, or indeed whether both are secondary to another common mechanism. The association of adrenocortical activity with electrolyte metabolism and the striking diurnal variation of adrenocortical activity (11) suggest that this may be the common trigger to muscle and kidney. Others (10) have suggested that the diurnal rhythm of potassium excretion is caused by cyclic changes in renal tubular metabolism with consequent changes in intracellular pH. These two suggestions are not mutually exclusive.

Carbohydrate metabolism of the forearm was studied in these subjects by measuring the net movement between forearm tissues and blood of glucose, lactate, oxygen and carbon dioxide simultaneously with potassium on the same samples of blood. Data on carbohydrate metabolism in the first 13 of these subjects have been reported previously (3, 4). The relation between movement of potassium and of these metabolites in the basal state was examined.

*Oxygen.* Since anoxia of muscle leads to loss of potassium, it might have been expected that subjects with the lowest $O_2$ consumption would have the greatest loss of potassium from muscle. However, the reverse was true (Figure 2).

*Lactate.* Lactate was produced by the resting forearm (mean = 0.44 μM per min. per 100 ml. forearm ± 0.11 S.E.M.). It is possible that there are small areas of muscle which are intermittently ischemic, perhaps as a result of rhythmic vasomotor activity, which accounts for this anaerobic metabolism. If so, correlation between lactate production by muscle and potassium loss from muscle would be expected. There was, however, no correlation.

*Glucose.* Glucose uptake by forearm tissue was small, averaging only 0.50 ± 0.17 μM per min. per 100 ml. forearm, and no correlation with potassium movement was present. The movement of potassium into cells along with glucose is generally attributed either to a) an increase in concentration within the cell of the potassium salt of the hexose phosphates or b) an increase of intracellular

fluid (and, therefore, of potassium) with glycogen deposition. With such a low uptake of glucose it would not be expected that either of these two processes occurred; in fact, breakdown of previously deposited glycogen would seem more likely and this might lead to a loss of associated potassium from muscle. Available data in the literature do not permit, however, an acceptable estimate of the quantity of glycogen breakdown required to account for the observed potassium movement. The mean R.Q. of the forearm in these subjects ($0.74 \pm 0.02$) suggests that glucose oxidation, and therefore glycogenolysis, must be minor and there is other evidence suggesting that little breakdown of glycogen in muscle is to be expected (3). There was also no correlation between movement of potassium and forearm R.Q.

*Carbon dioxide.* It has been demonstrated that acute acidosis, both respiratory and metabolic, leads to loss of potassium from muscle (12, 13). In the forearm experiments there was a highly significant correlation between $CO_2$ production by the forearm and net potassium movement (Figure 3). Greater $CO_2$ production does not, however, necessarily imply increased intracellular acidosis, since intracellular $CO_2$ concentration is a function not only of the rate of $CO_2$ production but also of the rate of $CO_2$ removal. $CO_2$ concentration in venous blood or the A-V difference in $CO_2$ concentration might then be better indices of tissue pH; neither of these correlated with the net movement of potassium. Since neither pH nor $pCO_2$ was measured, it is not possible from these experiments to be certain what role tissue pH might play.

In the three subjects studied during the night there was no correlation between movement of potassium and of any other metabolite.

## SUMMARY

There is net movement of potassium out of resting forearm muscle into plasma in the late morning hours in fasted subjects. From 1 A.M. to 10 A.M. no net movement occurs. From 10 A.M. to 1 P.M. the mean A-V difference in 21 subjects was $-0.23$ mEq. per L. The net loss in 15 subjects averaged 0.88 $\mu$Eq. per min. per 100 g. muscle, or about 0.5 per cent per hour of intracellular potassium.

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# MOVEMENT OF POTASSIUM INTO SKELETAL MUSCLE DURING SPONTANEOUS ATTACK IN FAMILY PERIODIC PARALYSIS [1]

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It has been appreciated since the classical studies of Biemond and Daniels (1) and Aitken, Allott, Castleden, and Walker (2) that the concentration of potassium in serum falls during attacks in patients with typical family periodic paralysis. Allott and McArdle (3), Pudenz, McIntosh, and McEachern (4) and Ferrebee, Atchley, and Loeb (5) showed that the quantity of potassium excreted in urine was reduced during attacks of paralysis. These observations implied that during attacks potassium shifted from extracellular to some intracellular space. Metabolic balance studies by Danowski, Elkinton, Burrows, and Winkler (6) confirmed this hypothesis. The question of which intracellular space accepted the potassium that shifted during attacks remained unanswered.

Recently it has been reported from this laboratory that there is a diurnal variation in normal man in exchange of potassium between skeletal muscle and extracellular water (7). During the hours between 10 P.M. and 12 noon, that is, 4 to 18 hours after the last meal, with the subject at rest, potassium moves from muscle to blood, the rate declining to a minimum or even reversing slightly (potassium moves from blood to muscle) between the hours of midnight and 7 A. M., and then rising again to a peak between 10 A.M. and noon. It is characteristic of family periodic paralysis that most attacks begin during the middle of the night (8). This suggested that in patients with this disease there may be an exaggeration of the diurnal variation in potassium movement, and that movement of potassium from extracellular fluid into skeletal muscle during the night might be responsible for the attacks. It is the purpose of this report to present data indicating that this is the case.

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## METHODS

Two subjects were studied. Both had well-documented family periodic paralysis with a history of spontaneous attacks of flaccid paralysis frequently having their onset in the middle of the night and associated with definite hypokalemia.

*Subject M. H.*, a 14-year-old boy weighing 57 Kg., had had episodes of weakness and paralysis since age 12. All the attacks began between 11 P.M. and 9 A.M. They were relieved or prevented by administration of potassium chloride, and serum potassium concentration during at least one attack was found to be low. The severity of his attacks was much less than that of the second patient.

Subject M. H. was studied on three occasions:

Study I was performed between 11:30 A.M. and 12 noon during spontaneous recovery from a mild attack which had begun spontaneously during the night. He had had no food since supper at 6 P.M. the evening prior to study.

Study II covered the hours between midnight and 7 A.M. The last meal was at 6 P.M. When a spontaneous attack did not occur by 2:20 A.M., an attack was induced by administration of glucose and of insulin.

Study III covered the hours between 1 A.M. and 7 A.M. During the afternoon prior to study M. H. exercised heavily on a treadmill and at 6 P.M. ate a large dinner. There was no clinical evidence of an attack during the time of this study.

*Subject A. B.*, a 24-year-old college student weighing 77 Kg., was a member of a family of whom six members had clinical evidence of periodic paralysis. His attacks began at age 12. There were one to three severe attacks and more frequent mild attacks each month until age 18 when he was first placed on prophylactic potassium therapy. Circumstances under which attacks occurred were quite predictable. Attacks followed heavy exercise and high carbohydrate meals, were aborted by mild exercise and ingestion of potassium chloride. Onset of attacks was usually in the middle of the night and some of his attacks were quite severe, involving not only the extremities but also respiratory muscles.

Subject A. B. was studied on one occasion. Prophylactic potassium therapy was discontinued 24 hours prior

TABLE I

*Net movement of potassium during spontaneous nocturnal attack—Subject A. B.**

| Time A.M. | A mEq./L. | A-V mEq./L. | Q̇ μEq./min./100 ml. forearm | Oral KCl g. | Muscle strength | Tendon reflexes† |
|---|---|---|---|---|---|---|
| 12:12 | 2.47 | 0.74 | 1.8 | | Normal | B-1; T-2 K-2, 2 |
| 1:08 | 2.11 | 0.60 | 1.3 | | | |
| 1:25 to 1:55 | | | | | Rapid decrease in arms; legs fairly strong. | B-0; T-0 K-1, 0; A-2, 1 |
| 2:08 2:55 | 2.11 | 0.55 | 0.9 | | Maximal inspiration reduced, but no dyspnea. Neck flexion very weak. | |
| 3:08 | 1.91 | 0.44 | 0.8 | | | |
| 4:00 | 1.85 | 0.49 | 1.1 | | Extremities almost totally paralyzed. (Severe EKG abnormalities.) | |
| 4:10 | | | | 5 | | |
| 4:40 | | | | 5 | Subjective improvement. No objective change. EKG less abnormal | B-0; T-1 K-0, 0; A-2, 1 |
| 5:48 | 2.75 | 0.61 | 0.8 | | | |
| 6:25 | 2.73 | 0.36 | 0.7 | 5 | Extremity strength slightly improved. Inspiration stronger. (EKG—return to severe changes.) | B-0; T-2 K-1, 0; A-2, 1 |
| 7:10 | | | | 5 | | |
| 7:13 | 3.11 | −0.20 | −0.4 | | Head can be raised off pillow. | |
| 7:43 | 3.45 | −0.50 | −0.8 | | | |
| 9:00 | 5.40 | −0.61 | −1.3 | | Nearly recovered. | B-2; T-1 K-3, 2; A-2, 2 |
| 10:03 | 5.91 | −0.33 | −0.7 | | Essentially normal. | |

*A = concentration of potassium in arterial plasma; A-V = arteriovenous difference in plasma concentration of potassium; Q̇ = net uptake (or net release when value is negative) of potassium by forearm tissues.

†B = biceps, T = triceps, K = knee, A = ankle. Only the right biceps and triceps reflexes could be tested; metabolic studies were made on the left arm. 0 = absent, 1 = reduced, 2 = normal, 3 = hyperactive. In the knee and ankle jerks, the first number refers to the right side, the second to the left side.

to this study. He received 100 g. of glucose at 3:30 P.M., ate his usual supper at 5:30 P.M. with extra dessert plus 100 g. of glucose at 6:30 P.M. Measurements were made between midnight and 10 A.M. A severe attack occurred and it was necessary to treat him with KCl.

Measurements were made of the metabolism of forearm muscles with the subject at rest. Uptake and release of potassium, $O_2$, $CO_2$, glucose and lactate were calculated as described previously (7, 9) as the products of forearm blood flow and arteriovenous differences of the metabolites. Venous blood was obtained through a catheter placed in a deep vein draining forearm muscles. Arterial blood was from the brachial artery. A pressure cuff about the wrist, inflated to greater than systolic pressure, excluded blood flow to hand and wrist during periods of blood collection.

Certain extrapolations of the data are made. In these it is assumed that uptake or output per min. per 100 ml. of forearm can be converted to uptake or output per min. per 100 g. of forearm muscle by multiplying by ⅘, a conversion factor estimated previously (9). It is further assumed that extracellular fluid weighs 20 per cent of body weight and that total muscle mass is 40 per cent of body weight.

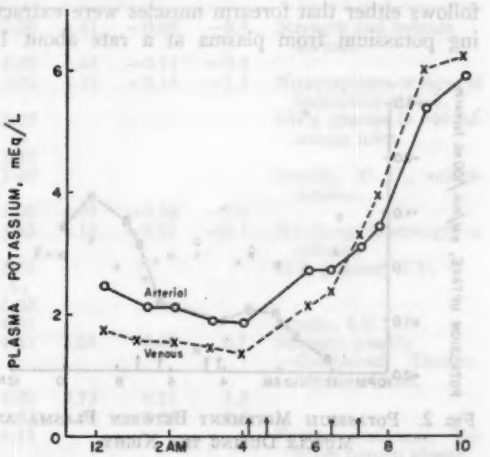


FIG. 1. ARTERIAL AND VENOUS PLASMA POTASSIUM CONCENTRATIONS DURING ATTACK AND RECOVERY IN PATIENT A. B.

At each time indicated by arrows 5 g. KCl was given orally.

## RESULTS AND DISCUSSION

### *Potassium Movement During Spontaneous Attack and Spontaneous Recovery*

*Subject A. B. (Table I and Figures 1 and 2)*

Arterial potassium concentration was already reduced greatly by the time the first sample was drawn at midnight and continued to fall for the next four hours, after which it was necessary to treat the patient by administration of KCl. In subject A. B., between midnight and 4 A.M., extraction of potassium (*i.e.*, A-V difference divided by arterial concentration) was approximately 25 per cent. In normal subjects little or no extraction of potassium occurs during these hours.

During the attack potassium moved from plasma into muscle at a rate of 1.2 $\mu$Eq. per min. per 100 ml. forearm. Between midnight and 4 A.M., concentration of potassium in arterial plasma fell 0.6 mEq. per L., a loss of about 9.5 mEq. of potassium from total extracellular fluid. During that time about 2 mEq. of potassium moved from plasma into muscles of one forearm. If all skeletal muscles were behaving identically, about 110 mEq. of potassium might have moved from extracellular fluid into muscle in the four-hour period. Since this figure is about 12 times as large as the estimated loss of extracellular potassium it follows either that forearm muscles were extracting potassium from plasma at a rate about 12 times greater than the average muscle or that potassium was added to plasma from some nonmuscular source.

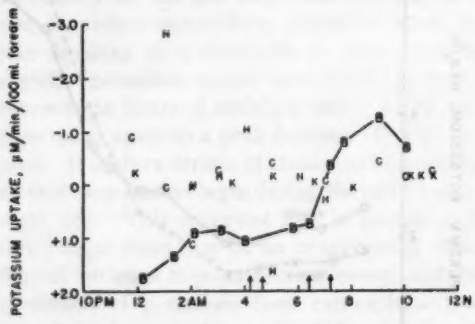


FIG. 2. POTASSIUM MOVEMENT BETWEEN PLASMA AND MUSCLE DURING THE NIGHT

Positive values are uptake, negative values are release from muscle. B, subject A. B. during attack and recovery; K and C, normal subjects; H, subject M. H. who had periodic paralysis but failed to get an attack during this study. Arrows indicate oral administration of 5 g. KCl to A. B.

Although the mass of potassium lost from extracellular fluid was large from the viewpoint of extracellular potassium, if it all went into skeletal muscle this quantity of potassium would be sufficient to raise the average concentration of intramuscular potassium by only a few per cent, an amount too small to be detected by available methods.

When KCl was administered, although there was some elevation of arterial potassium concentration, extraction of potassium remained constant for the next two and one-half hours and potassium continued to move into muscle until the direction of movement was reversed at 7 A.M., several hours earlier than the onset of accelerated output of potassium by muscle in normal subjects.

In summary, an attack of paralysis developed in the middle of the night about seven hours after a heavy carbohydrate meal. Potassium moved from plasma into skeletal muscle, concentration of potassium in plasma was reduced and paralysis occurred. Efforts to terminate the attack by oral administration of potassium failed to do so during a two-hour period in which potassium continued to move from plasma into muscle and there was only a small increase in concentration of arterial potassium. Presumably, during these two hours potassium movement out of extracellular space into muscle almost kept pace with intestinal absorption of administered potassium. Later in the morning the direction of potassium movement reversed; potassium moved out of muscle into extracellular space at a rate greater than that seen in normal subjects during these hours. Concentration of potassium in arterial plasma rose abruptly and the attack ended. Although the rise in concentration of potassium in arterial plasma may have been the result of absorption of administered potassium from the gut as well as of outpouring of potassium from skeletal muscle, the latter factor alone was sufficient to account for the observed rise in plasma concentration.

*Subject M. H., Study I (Table II, Figure 3)*

Subject M. H. failed to have a spontaneous attack during the time measurements of forearm

TABLE II

*Potassium release during spontaneous recovery from spontaneous attack—Subject M. H.**

| Time | A | A-V | Q̇ |
|---|---|---|---|
| 11:39 | 4.09 | −1.05 | −3.85 |
| 11:50 | 4.10 | −1.15 | −6.23 |
| 12:00 | 4.08 | −0.87 | −3.70 |

* Time is A.M. Symbols as in Table I.

metabolism were made. However, forearm metabolism was measured on the morning following a mild spontaneous attack from which he was recovering spontaneously. At the time of the study there was no longer any gross weakness. Deep tendon reflexes in the arms were normal but patellar and ankle responses were still slightly depressed. Release of potassium from muscle was about six times greater than the average rate found in normal subjects during the same time of the day. Presumably at some earlier hour during the attack there was a reduction in plasma potassium concentration. The rate at which potassium moved from muscle to extracellular fluid during recovery, 3 to 5 mEq. per hr. per Kg. of muscle, was adequate to account for an increase in plasma potassium concentration. Despite the large contribution of potassium to venous plasma from muscle, concentration of potassium in arterial plasma was stable at about 4 mEq. per L. during the period of observation. This stability of arterial potassium could obtain only if potassium left the blood stream by some other route as fast as it entered from skeletal muscle.

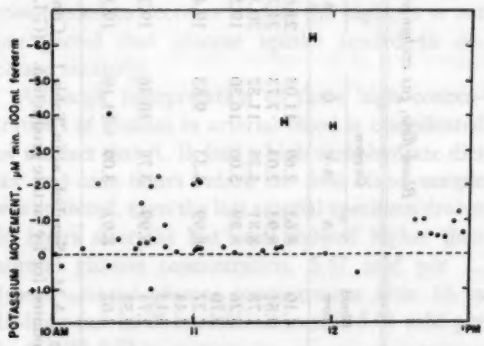


FIG. 3. POTASSIUM OUTPUT FROM MUSCLE TO PLASMA DURING SPONTANEOUS RECOVERY FROM A SPONTANEOUS ATTACK OF PARALYSIS IN SUBJECT M. H.

●, data from normal subjects in the basal state.

### *Potassium Movement During an Attack Produced by Administration of Glucose and Insulin*

### *Subject M. H., Study II (Table III)*

During Study II of subject M. H., when a spontaneous attack had not occurred by 2:20 A.M. a mild attack was produced by administration of glucose and insulin. At 6:25 A.M. he was given 9 g. KCl by mouth. Within 25 minutes there was some return of deep tendon reflexes.

When KCl was administered there was a rapid rise in arterial potassium concentration despite a large increase in rate of potassium uptake by muscle. An uptake of this magnitude did not occur following potassium administration to subject A. B. at approximately the same time of day. The difference in response is probably attributable to the effect of insulin, since the behavior of blood glucose concentrations indicated that a potent insulin action occurred at the time of massive uptake of potassium.

From the rise in concentration of potassium in

TABLE III

*Net movement of potassium during attack induced by glucose and insulin—Subject M. H., Study II **

| Time A.M. | A mEq./L. | A-V mEq./L. | Q̇ μEq./min./100 ml. forearm | Comments |
|---|---|---|---|---|
| 12:20 | 4.41 | −0.06 | −0.4 | Strength and reflexes normal. |
| 1:20 | 4.44 | −0.11 | −0.8 | |
| 2:21 | 4.31 | −0.16 | −1.2 | No symptoms or signs of impending attack. |
| 2:25 to 2:40 | | | | 130 g. glucose in 240 ml. orange juice. |
| 2:40 | | | | Insulin, 20 U., subcutaneous. |
| 3:02 | 4.09 | −0.34 | −2.6 | |
| 3:55 | 4.18 | −0.01 | −0.1 | No change in strength or reflexes. |
| 3:55 to 4:05 | | | | 25 g. glucose, I.V. |
| 4:07 | | | | Insulin, 6 U., I.V. |
| 4:41 | 3.68 | 0.08 | 0.7 | Strength greatly diminished. Tendon reflexes absent. |
| 5:29 | 3.73 | 0.26 | 1.8 | |
| 6:03 | 3.68 | 0.03 | 0.3 | |
| 6:15 | | | | Further weakness. Reflexes remain absent. |
| 6:25 | | | | 9 g. KCl orally. |
| 6:40 | 4.95 | 0.68 | 6.6 | Reflexes and strength returning. |
| 6:58 | 5.56 | 0.45 | 5.7 | Almost complete recovery. |

* Symbols as in Table I.

arterial plasma, from the measured uptake of potassium by muscles of the forearm, and from reasonable assumptions of the size of extracellular space and total muscle mass in M. H. it can be estimated that of the 120 mEq. of potassium administered about 20 mEq. went into extracellular space and about 65 mEq. went into skeletal muscle in 33 minutes, leaving about one-fourth unaccounted for. Similar calculations made for the case of A.B. following the first 10 g. of administered KCl (Table I) indicated that of the 135 mEq. of potassium administered about 14 mEq. went into extracellular space and about 35 mEq. went into muscle in two hours, leaving about two-thirds of the potassium unaccounted for. This suggests that the failure of oral administration of KCl to produce prompt restoration of normal potassium concentration in arterial plasma and clinical recovery may have been in part due to delayed absorption of potassium from the gut during the more severe attack suffered by A. B.

### *Normal Pattern of Potassium Movement During a Night When No Paralytic Attack Occurred*

*Subject M. H., Study III (Figure 2)*

During the early part of Study II, between midnight and 2:20 A.M., subject M. H. had no spontaneous attack and potassium movement at this time was indistinguishable from that observed in normal men. In Study III, between 1 A.M. and 7 A.M., subject M. H. again had no spontaneous attack and potassium movement appeared to be normal.

### *Fragmentary Observations on Other Abnormalities of Muscle Metabolism Occurring During a Spontaneous Attack of Paralysis (Table IV)*

Certain observations discovered during the attack in A. B. which differ from those obtained in normal subjects are reported. These differences may prove ultimately to be of no significance since the number of normal subjects studied is small. On the other hand, even if they prove to be real departures from the normal it is not clear whether these differences are fundamental to the disease, whether they represent a defect parallel to but unrelated to the anomaly of potassium movement, whether they are in some way causally related to

TABLE IV

*Blood flow, gas exchange and carbohydrate metabolism in muscle during spontaneous paralysis—Subject A. B.**

| Time | Blood flow | Oxygen | | | Carbon dioxide | | | R.Q. | Glucose | | | Lactate | | | | (G-L)/$O_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | A-V | $\dot{Q}$ | A | V-A | $\dot{Q}$ | | A | A-V | $\dot{Q}$ | A | A-V | $\dot{Q}$ | L/G | |
| 12:12 | 4.10 | 8.62 | 2.69 | 11.03 | 21.61 | 2.48 | 10.17 | 0.92 | 6.42 | 0.98 | 4.02 | 0.595 | −0.139 | −0.570 | 7 | 203 |
| 1:08 | 3.81 | 8.93 | 2.03 | 7.73 | 21.28 | 2.99 | 11.39 | 1.47 | 6.47 | 0.56 | 2.13 | 0.655 | 0.071 | 0.271 | None | 176 |
| 2:08 | 2.78 | 8.81 | 4.25 | 11.82 | 20.33 | 4.05 | 11.26 | 0.95 | 6.06 | 0.28 | 0.78 | 0.517 | −0.056 | −0.156 | 10 | 36 |
| 3:08 | 3.26 | 8.89 | 5.06 | 16.50 | 19.58 | 4.57 | 14.90 | 0.90 | 6.38 | 0.50 | 1.63 | 0.538 | −0.134 | −0.437 | 13 | 51 |
| 4:00 | 3.70 | | | | | | | | | | | | | | | |
| 5:48 | 2.27 | 8.99 | 4.33 | 9.83 | 19.49 | 3.83 | 8.69 | 0.88 | 5.77 | −0.10 | −0.23 | 0.772 | 0.038 | 0.086 | All | −16 |
| 6:26 | 3.44 | | | | | | | | | | | | | | | |
| 7:13 | 3.13 | | | | | | | | | | | | | | | |
| 7:43 | 2.74 | 8.99 | 3.78 | 10.36 | 19.34 | 3.47 | 9.51 | 0.92 | 5.80 | 0.12 | 0.33 | 1.048 | 0.215 | 0.589 | None | 36 |
| 9:00 | 3.55 | | | | | | | | | | | | | | | |
| 10:03 | 3.62 | 8.92 | 3.09 | 11.19 | 19.39 | 2.30 | 8.33 | 0.74 | 5.57 | 0.21 | 0.76 | 0.767 | −0.025 | −0.090 | 6 | 38 |

* Time is A.M. Symbols as in Table I. Concentrations are in mM per L. $\dot{Q}$ expressed as μM per min. per 100 ml. forearm. L/G is per cent of glucose uptake accounted for by lactate production. (G-L)/$O_2$ is per cent of $O_2$ uptake accounted for by apparent oxidation of glucose.

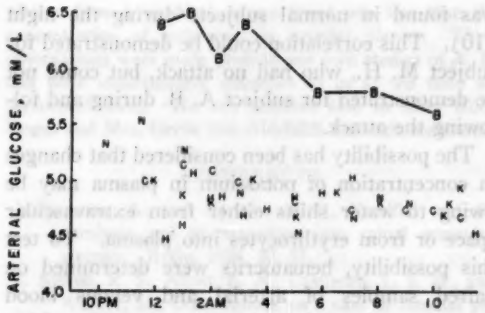


FIG. 4. ARTERIAL BLOOD GLUCOSE

B, subject A. B. during attack and recovery; C, K and N, normal subjects; H, subject M. H. who failed to develop hypokalemia or clinical paralysis.

potassium movement, or whether they are consequences of the disease. None of these metabolic differences appeared in subject M. H., Study III, during the night in which he failed to have a spontaneous attack.

Concentration of glucose in arterial blood was definitely higher in subject A. B. than in normals during the night (Figure 4), and at least during the first four measurements A-V glucose differences were higher than normal. It has been noted previously (10) that A-V glucose differences varied directly with arterial glucose concentrations in normal subjects during the night. This relation was true also for subject A. B., so that the abnormally high glucose A-V differences and glucose uptakes in this subject may have resulted from abnormally high delivery of glucose to muscle. Since the concentration of arterial glucose tended to decrease during the night, it is not unexpected that glucose uptake tended to decrease similarly.

Although interpretation of these high concentrations of glucose in arterial blood is complicated by the fact that A. B. had a high carbohydrate diet six and nine hours before the first blood sample was collected, even the last arterial specimen drawn 16 hours after the last meal showed higher than normal glucose concentration, 5.57 mM per L. (mean arterial glucose concentration after 16 to 18-hour fast in 24 normal subjects is 5.01 mM per L. ± 0.27 S.D.).

Since uptake of glucose by skeletal muscle was at least normal, the high concentration of glucose in arterial blood implies that some other organ failed to remove circulating glucose or that hepatic contribution of glucose was accelerated.

Concentration of lactate in arterial blood was within normal limits during the early part of the study and rose during the later hours (Figure 5), approximately coincident with recovery from paralysis. Lactate production by muscle was erratic. There is no evidence that the rise in arterial lactate concentration was owing to increased production of lactate by skeletal muscle in the forearm. It is possible, however, that other muscles did contribute, since muscle power and deep tendon reflexes were examined from time to time during this period.

During the night and the following morning, forearm blood flow in subject A. B. was within the limits defined by studies of two normal subjects during the night (10) and a larger group of normal subjects during the late morning (9). Nor were there any deviations from normal in $O_2$ uptake, in $CO_2$ production or in A-V differences of $CO_2$ or $O_2$ content. However, the arterial concentration of $CO_2$, initially normal, fell to levels lower than those in normal subjects (mean of 24 normal subjects, 21.9 mEq. per L. ± 0.98 S.D.) (Figure 6). Although pH was not measured, this decrease in arterial $CO_2$ presumably represented extracellular metabolic acidosis.

Mean respiratory quotients of forearm muscles in three normal subjects between the hours of 10 P.M. and 4 A.M. were 0.75, 0.84 and 0.84. In subject M. H., during the same time interval

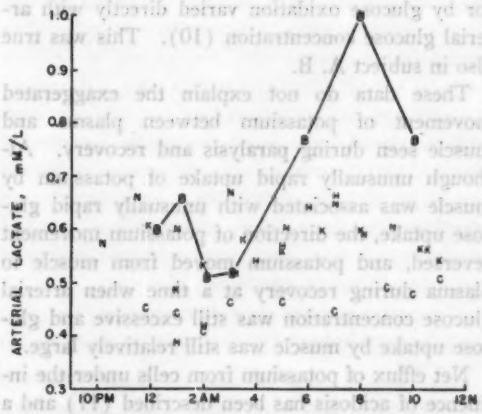


FIG. 5. ARTERIAL BLOOD LACTATE

Symbols as in Figure 4.

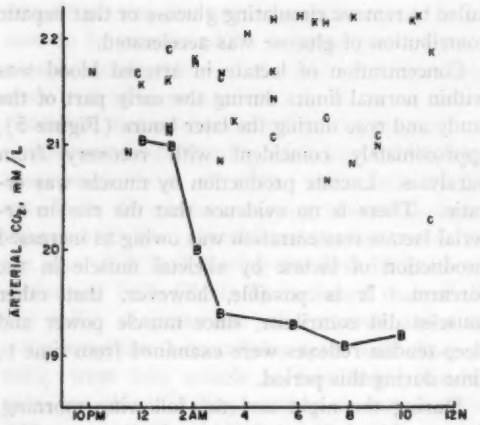


FIG. 6. ARTERIAL BLOOD $CO_2$ CONTENT
Symbols as in Figure 4.

when he failed to have an attack, the R.Q. was 0.82. In subject A. B. during the spontaneous attack, R.Q. was 1.00.

The fraction of $O_2$ uptake by muscle which is spent in oxidation of glucose is defined by the following relation (9): 6(glucose A-V difference minus ½ lactate A-V difference)/oxygen A-V difference. In contrast to data found in normal subjects during the same hours, during the early hours of the attack glucose uptake was greatly in excess of the amount needed to account for all the $O_2$ uptake. Later, glucose uptake decreased and was sufficient to account for only a minor fraction of $O_2$ uptake, as was true in normals. In normal subjects the fraction of $O_2$ uptake accounted for by glucose oxidation varied directly with arterial glucose concentration (10). This was true also in subject A. B.

These data do not explain the exaggerated movement of potassium between plasma and muscle seen during paralysis and recovery. Although unusually rapid uptake of potassium by muscle was associated with unusually rapid glucose uptake, the direction of potassium movement reversed, and potassium moved from muscle to plasma during recovery at a time when arterial glucose concentration was still excessive and glucose uptake by muscle was still relatively large.

Net efflux of potassium from cells under the influence of acidosis has been described (11) and a suggestive correlation between potassium movement from muscle and $CO_2$ production by muscle was found in normal subjects during the night (10). This correlation could be demonstrated for subject M. H., who had no attack, but could not be demonstrated for subject A. B. during and following the attack.

The possibility has been considered that changes in concentration of potassium in plasma may be owing to water shifts either from extravascular space or from erythrocytes into plasma. To test this possibility, hematocrits were determined on paired samples of arterial and venous blood throughout the night. Among seven pairs of hematocrits, mean arterial hematocrit was 44.20, range 43.1 to 45.5, mean venous hematocrit was 44.26, range 43.4 to 45.5, and mean difference between pairs of arterial and venous blood was − 0.06, range − 0.5 to + 1.0. Thus there was no evidence of net water shift into plasma.

## SUMMARY

1. A large net uptake of potassium from arterial plasma by skeletal muscle (the forearm) has been demonstrated a) during the development of a spontaneous nocturnal attack of periodic paralysis and b) during the development of a nocturnal attack induced by the administration of glucose and insulin.

2. Movement of potassium out of skeletal muscle has been demonstrated a) during the spontaneous recovery from a nocturnal attack which developed spontaneously and b) during recovery from an attack after oral KCl administration.

3. The characteristic onset of attacks during the night with spontaneous cure later in the morning seen in many of these patients may be due to exaggerations of the normal diurnal variation in net potassium movement between muscle and plasma.

## ACKNOWLEDGMENTS

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plasma from antecubital venous blood was less than the concentration of potassium in arterial plasma. Their observations were made prior to our own studies of A. B. and were unfortunately unknown to us at the time we performed them. We are indebted also to Miss Ellen Rogus and Mrs. Gerda von Ahlefeldt for their assistance.

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# THE ULTRAFILTRABLE CALCIUM OF HUMAN SERUM. I. ULTRAFILTRATION METHODS AND NORMAL VALUES [1]

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Since the demonstration by Rona and Takahashi in 1911 (1) that a considerable portion of the calcium present in the serum is not diffusible through a semi-permeable membrane, the various calcium-fractions in the blood have been extensively studied. It is now recognized that calcium exists in the serum in three distinct forms. One of these, that calcium bound to proteins, comprises the non-ultrafiltrable or non-diffusible portion of the serum calcium. The other two forms, ionic calcium and calcium complexed by such small anions as citrate, phosphate, and bicarbonate, are ultrafiltrable and diffusible.

Ionic calcium is generally considered to be the physiologically active component of the total serum calcium (20). Recently, however, it has been suggested (21) that the "biologically active" calcium fraction of serum is different from, and probably larger than, the total ionic calcium as determined by the frog heart technique (22), approaching the value for total ultrafiltrable calcium. In any event, a practical method for the routine measurement of actual ionic calcium has yet to be devised. Consequently, a great deal of effort has been directed toward the development of indirect methods for its determination in man. A variety of techniques has been described, but ultrafiltration methods have been most numerous because of practical and theoretical advantages over biological assays or dialysis techniques. In principle, all ultrafiltration procedures utilize some type of membrane of small pore size and some means of supplying filtration pressure.

The purpose of the present communication is to describe a technique for the ultrafiltration of human serum using a new, simple apparatus[2] which eliminates most of the disadvantages of previous methods. Using this apparatus we have investigated the various factors which influence the ultrafiltrability of calcium in human serum and have determined the normal range in healthy human subjects. A subsequent paper will describe our findings in diseased states and under experimental conditions.

## METHODS

*Ultrafiltration apparatus and procedure.* The apparatus used in this work has been described very briefly in a previous publication (23). It uses seamless cellophane tubing to contain the serum with a sintered glass support for the membrane and centrifugal force to supply the filtration pressure. Its principal advantages over other equipment are: 1) The atmosphere and the pH can be accurately controlled within the apparatus throughout the ultrafiltration period; 2) there is no source of metallic contamination; 3) membrane breakage is virtually eliminated; 4) the apparatus is easily constructed and can be used in an ordinary laboratory centrifuge; 5) filtration pressure can be controlled by varying centrifuge speed.

The ultrafiltration procedure was carried out in the following manner:

A strip of Visking Nojax Casing® (size 24/32) about 9 inches long was soaked in distilled water for 10 minutes. It was wiped dry by drawing through a folded gauze sponge repeatedly until no water was visible. One end was knotted and the tubing was doubled (Figure 1) and pushed into the ultrafiltration tube with the knot up. The unknotted end was opened with a sharp instrument such as a scalpel blade or small spatula and inflated by blowing into it. For the present studies, 3 ml. of serum were pipetted directly into the open end of the bag. Care was taken to avoid contact between the wet

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

[2] Made by sealing off the short end of a borosilicate glass straight sealing tube with coarse fritted disc (Corning No. 39570, 25-mm. diameter with 20-mm. disc), and adding a 6-mm. glass tube at an angle near the fritted disc (Figure 1). Complete apparatus is now available from Will Corporation, Rochester, New York.

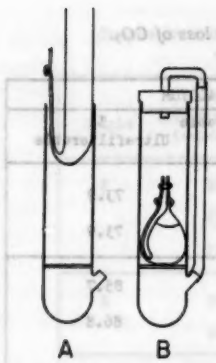


FIG. 1. THE APPARATUS USED FOR ULTRAFILTRATION OF SERUM

A. The apparatus containing the knotted and inflated Visking® tubing prior to the introduction of the serum sample.

B. The apparatus closed and ready for centrifugation showing the serum filled ultrafiltration sac resting on the sintered glass support.

tip of the pipet and the cellophane tubing because such contact resulted in difficulty in removing the pipet. After the serum was introduced, the cellophane bag was collapsed, and the second end was knotted at about the same height as the first end. The two knotted ends were fastened together with a rubber band, and the bag was carefully pushed down into the apparatus far enough to permit insertion of the rubber stopper. To avoid tearing the bag, a blunt instrument, such as a stirring rod, was used to push adherent portions of the bag away from the glass. The apparatus was then flushed for 3 minutes with a 5 per cent $CO_2$–95 per cent $O_2$ mixture bubbled through isotonic saline and introduced through the side arm at the lower end of the apparatus. The apparatus was then closed by connecting the side arm to the glass tube in the rubber stopper at the top using Tygon® tubing, thus assuring a constant atmosphere above and below the ultrafiltration sac throughout the ultrafiltration procedure (Figure 1). It was then placed in the largest cups (catalog No. 373) of an International Centrifuge, Size 2, or International Refrigerated Centrifuge, the cups having been fitted with the simple centering ring previously described (23) so that apparatus breakage was prevented while starting and stopping the centrifuge.

A temperature rise of about 8° C above the existing room temperature will occur inside the operating centrifuge in about 1 to 2 hours. To keep the temperature constant during ultrafiltration in the ordinary laboratory centrifuge, the latter was fitted with a copper coil. This was connected with Tygon® tubing to the side-arm of a large glass U-tube. One arm of the U-tube was connected to the ordinary mixing-type water spigot and the other was fitted with a thermometer. Inlet water temperature was manually controlled by simply varying the amount of hot and cold water allowed to flow through the spigot into the U-tube. A thermometer was fitted into the center tachometer hole of the centrifuge and the temperature inside the centrifuge was kept constant by the adjustments in the inlet water temperature.

At 23° or 37° C, at least 0.5 ml. of a crystal-clear protein-free (24) ultrafiltrate was collected in 3 hours when centrifuged at 2,000 rpm, while at 10° C approximately double that time was necessary. If 5 ml. or more of serum were used, 1 ml. of ultrafiltrate could be obtained in approximately the same times. Variations in centrifuge speed between 1,500 and 2,500 rpm do not affect the results obtained (24).

*Determination of calcium.* Calcium in both serum and ultrafiltrate was determined by a flame photometric technique using a Weichselbaum-Varney flame photometer. After protein removal (in the case of serum) the calcium was separated as the oxalate, re-dissolved, and read directly. A description of the method together with a thorough study of possible variables have already been reported (25).

*Determination of pH.* A Beckman model G pH meter was used for the pH measurements. In all instances, the pH of the serum remaining in the ultrafiltration sac was measured after the ultrafiltration was carried out. In several instances, pH values of both the ultrafiltrate and the unfiltered serum residue were determined. As would be expected where the atmosphere was maintained constant over the serum and ultrafiltrate, the pH values were the same. Generally, the value obtained was between 7.5 and 7.6 if the apparatus were allowed to return to room temperature (at which the equilibrium with 5 per cent $CO_2$–95 per cent $O_2$ mixture was carried out) and the pH measurement were then made.

Early in the present work it was noted that the pH tended to drift with time as the concentrated serum left in the ultrafiltration sac was being measured with a conventional asbestos-fibre calomel electrode. This was virtually eliminated, however, when a sleeve-type calomel electrode (Beckman No. 270–71) was used.

*Collection of sample.* Blood was collected with an ordinary syringe and a 20-gauge needle by venipuncture in the antecubital fossa. No precautions were taken to prevent loss of $CO_2$ (see discussion under *Effect of method of sample collection*). The ultrafiltration procedure was carried out on the same day the samples were collected.

## RESULTS

In expressing ultrafiltration results, some authors have used a corrected serum volume calculated from the estimated volume occupied by the proteins present. We have not used the correction but have applied the following definition to express our results:

$$\% \text{ Ultrafiltrable Ca} = \frac{\text{Mg. Ca/100 ml. ultrafiltrate}}{\text{Mg. Ca/100 ml. serum}} \times 100$$

TABLE I

*Results obtained with and without precautions for loss of $CO_2$. Ultrafiltration temperature 23° C*

| Sample | Method | pH | | SERUM CALCIUM | | |
|---|---|---|---|---|---|---|
| | | Initial | Final | Total mg% | Ultrafilterable mg% | % Ultrafilterable |
| R | oil | 7.52 | 7.47 | 9.52 | 7.04 | 73.9 |
| | plain | 7.72 | 7.51 | 9.52 | 7.04 | 73.9 |
| F* | oil | 7.52 | 7.51 | 12.1 | 10.35 | 85.7 |
| | plain | 7.82 | 7.52 | 12.1 | 10.48 | 86.8 |

* Patient with carcinoma of breast and hypercalcemia.

*Effect of method of sample collection*

It has been reported by Hopkins, Howard, and Eisenberg (18) that when serum is equilibrated with 5 per cent $CO_2$–95 per cent $O_2$ to adjust pH before ultrafiltration, the method of collection of the blood is not particularly important. Prasad and Flink (19), on the other hand, considered the method of collection and equilibration of serum highly important.

Our findings are in agreement with those of Hopkins, Howard, and Eisenberg and are shown in Table I. Blood allowed to clot under oil and transferred without loss of $CO_2$ gave results identical with those obtained when no special precautions were exercised, provided the apparatus was flushed with $CO_2$–$O_2$ mixture before the ultrafiltration procedure was carried out.

*Effect of duration of ultrafiltration on composition of ultrafiltrate*

Since ultrafiltration is a dynamic process and concentrates the serum proteins, a criticism frequently voiced is that the calcium concentration in the ultrafiltrate may not reflect the original conditions in the whole serum. This criticism may be shown to be invalid by the application of physical chemical principles to the finding of Marrack and Thacker (26) and McLean and Hastings (22) that the relationship between calcium and serum proteins may be expressed by the simple equation: $Ca^{++} + Prot^{=} \rightleftharpoons$ CaProteinate.[8] Where a 1:1 relationship between the calcium and protein in the complex exists, these theoretical considerations show that the composition of the ultrafiltrate

[8] The thermodynamic dissociation constant of the calcium complex is

$$K = [Ca^{++}] \cdot \frac{[Prot^{=}]}{[Ca\ Proteinate]}$$

where the quantities in the brackets are activities. According to this equation the ratio of bound to unbound protein is a function of the calcium ion activity in the solution. In serum of ionic strength 0.15 M, the protein concentration is about 0.001 M and is considered to contribute a negligible amount to the total ionic strength (27). Thus relatively large alterations in protein concentration must be produced before any change in ionic strength occurs. Chen and Neuman (24) showed that diffusible forms of calcium (calcium chloride, calcium citrate, and calcium versenate) in 0.15 M sodium chloride move through a cellophane membrane at the same rate as water. Similar behavior during ultrafiltration of serum would then cause no change in the concentration of any diffusible ions in the free water of the bulk solution, an increase in the concentration of both forms of protein, but only a negligible change in ionic strength. Since the largest factor in the determination of activity coefficients is the ionic strength, the activity coefficients of all constituents in the remaining serum would remain essentially constant during the ultrafiltration process. Thus, with no change in activity coefficients, calcium ion concentration and the ratio of the two forms of protein do not change.

The above discussion would apply equally to any reaction involving a fixed number of one or more calcium ions with a single protein molecule. Thus

$$n\ Ca^{++} + Prot \rightleftharpoons Ca_n\ Prot$$

$$K = [Ca^{++}]^n \frac{[Prot]}{[Ca_n\ Prot]}$$

The ultrafiltration procedure would not change the $Ca^{++}$ concentration and the Prot/$Ca_n$ Prot ratio would remain constant although the concentrations of the two forms of protein change continuously.

TABLE II

*Successive samples during prolonged ultrafiltration. Patients with chronic renal disease and hypocalcemia*

| Sample | Ultrafiltration temperature | Time in hours | SERUM CALCIUM | | |
|---|---|---|---|---|---|
| | | | Total mg% | Ultrafilterable mg% | % Ultrafilterable |
| E | 23° | 0- 4 | 8.86 | 7.17 | 80.9 |
| | 23° | 4- 7 | 8.86 | 7.17 | 80.9 |
| | 23° | 7-20 | 8.86 | 6.90 | 77.9 |
| R | 36° | 0- 3 | 5.58 | 3.81 | 68.3 |
| | 36° | 3- 7 | 5.58 | 3.81 | 68.3 |
| C | 23° | 0- 3 | 7.45 | 5.72 | 76.7 |
| | 23° | 3- 7 | 7.45 | 5.72 | 76.7 |
| | 36° | 0- 3 | 7.45 | 5.04 | 67.7 |
| | 36° | 3- 7 | 7.45 | 5.17 | 69.4 |

should be that of the non-protein portion of the original serum and should not change unless the ionic strength of the solution being ultrafiltered changes significantly during the process. These considerations were confirmed by collecting successive samples of ultrafiltrate during prolonged ultrafiltration of a single 3-ml. aliquot of serum and determining the calcium content of the samples.

TABLE III

*Variability of ultrafilterable calcium with pH*

| Sample | Ultrafiltration temperature | Ultrafiltrate pH | SERUM CALCIUM | | |
|---|---|---|---|---|---|
| | | | Total mg% | Ultrafilterable mg% | % Ultrafilterable |
| R | 36° | 7.68 | 9.51 | 6.41 | 67.4 |
| | 36° | 8.22 | 9.51 | 5.28 | 55.6 |
| B | 36° | 6.35 | 9.40 | 8.05 | 86.8 |
| | 36° | 7.51 | 9.40 | 6.18 | 66.7 |
| J | 36° | 6.35 | 9.27 | 8.60 | 91.5 |
| | 36° | 8.18 | 9.27 | 5.38 | 57.3 |
| M* | 36° | 7.68 | 11.57 | 8.34 | 72.2 |
| | 36° | 8.22 | 11.57 | 6.86 | 59.3 |
| F | 23° | 6.52 | 10.15 | 9.28 | 91.4 |
| | 23° | 7.63 | 10.15 | 7.76 | 76.4 |
| | 23° | 8.40 | 10.15 | 5.82 | 57.3 |
| S | 23° | 7.56 | 9.89 | 7.48 | 75.7 |
| | 23° | 8.37 | 9.89 | 5.67 | 57.3 |
| M* | 10° | 7.44 | 11.57 | 9.30 | 80.3 |
| | 10° | 8.14 | 11.57 | 8.13 | 70.3 |

* Patient with carcinoma of the breast and hypercalcemia.

**TABLE IV**

*Comparison of acid and carbon dioxide pH adjustment. Ultrafiltration temperature 23° C*

| Method of Adjustment | Adjusted pH | SERUM CALCIUM | | |
|---|---|---|---|---|
| | | Total mg% | Ultrafilterable mg% | % Ultrafilterable |
| 5% $CO_2$–95% $O_2$ | 7.55 | 8.14* | 6.64 | 81.6 |
| 100% $CO_2$ | 6.48 | " | 7.58 | 93.2 |
| Hydrochloric acid | 6.58 | " | 7.49 | 92.1 |

* Hypocalcemia secondary to renal disease.

The ultrafiltration apparatus was re-equilibrated with the $CO_2$–$O_2$ mixture after each 0.6-ml. sample was collected for analysis and the results are shown in Table II.

From an original sample of 3 ml. the collection of approximately 0.6 ml. of ultrafiltrate for calcium analysis will result in a reduction in volume of 20 per cent in the original serum or an increase in the concentration of protein of 25 per cent. In the first sample shown in the table the three successive 0.6-ml. aliquots of ultrafiltrate decreased the original serum volume to less than one-half of the original volume. Thus a large fraction of the serum may be ultrafiltered without an appreciable change in the composition of the ultrafiltrate, and the reduction in volume of the serum accompanying the usual ultrafiltration would not be expected to affect the results obtained.

### *Effect of pH*

The literature contains conflicting information on this subject. Almost without exception, early

**TABLE V**

*Effect of temperature on ultrafilterability of calcium*

| Sample | Ultrafiltration temperature | SERUM CALCIUM | | |
|---|---|---|---|---|
| | | Total mg% | Ultrafilterable mg% | % Ultrafilterable |
| J | 3.5° | 9.36 | 7.96 | 85.1 |
| | 10 | 9.36 | 7.41 | 79.2 |
| | 23 | 9.36 | 6.96 | 74.3 |
| | 36 | 9.36 | 6.51 | 69.6 |
| B | 3.5° | 10.27 | 7.46 | 72.6 |
| | 10 | 10.27 | 7.24 | 70.5 |
| | 23 | 10.27 | 6.96 | 67.8 |
| | 36 | 10.27 | 6.24 | 60.8 |
| R | 10° | 9.84 | 7.81 | 79.3 |
| | 23 | 9.84 | 7.33 | 74.6 |
| | 36 | 9.84 | 6.44 | 65.5 |
| T | 10° | 9.74 | 7.46 | 76.6 |
| | 23 | 9.74 | 7.26 | 74.5 |
| | 36 | 9.74 | 6.58 | 67.7 |

workers took no special precautions to insure constancy of pH during the process of ultrafiltration. It has been reported (2, 3, 10) that variations in pH during ultrafiltration have no effect on the results obtained although it was admitted that, on theoretical grounds, this was unexpected (10). In any event, rigid control of pH would have been difficult since the methods most frequently utilized (Greenberg-Gunther [10] and Moritz-Updegraff [6]) had no adequate provision for maintaining a constant atmosphere to control pH throughout the entire ultrafiltration process (29). Recently, however, Hopkins, Howard, and Eisenberg (18), using the closed apparatus of Lavietes (28), reported a very definite effect of pH on the fraction of serum calcium appearing in the ultrafiltrate.

The apparatus used in the present study also controls the atmosphere rigidly during the filtration process and we have reinvestigated the problem. Our data, recorded in Table III, confirm the observation that the pH at which ultrafiltration is carried out affects significantly the fraction of calcium that is ultrafiltrable. Analysis of these data shows that the per cent of ultrafiltrable calcium changed from 1.5 to 2.5 per cent per 0.1 pH unit in good agreement with values calculated from data previously reported (26, 18, 30).

The lowering of pH was accomplished by equilibrating the serum in the ultrafiltration apparatus with 100 per cent $CO_2$. The high pH's were obtained by ultrafiltering serum with no carbon dioxide equilibration. Hopkins, Howard, and Eisen-

TABLE VI

*Ultrafilterable calcium values for normal human serum at various temperatures. (All samples equilibrated with 5 per cent $CO_2$—95 per cent before ultrafiltration)*

| Sample | Sex | Ultrafiltration temperature | Total Calcium* | Ultrafilterable Calcium* | % Ultrafilterable |
|---|---|---|---|---|---|
| W.N. | M | 36° | 9.86 | 6.54 | 66.2 |
| J.W. | M | | 9.73 | 6.38 | 65.6 |
| H.F. | M | | 10.30 | 6.27 | 60.9 |
| R.T. | M | | 9.60 | 6.20 | 64.6 |
| T.T. | M | | 9.26 | 6.38 | 68.9 |
| B.B. | M | | 10.22 | 7.16 | 70.0 |
| E.L. | M | | 9.53 | 6.80 | 71.4 |
| H.G. | M | | 10.12 | 6.17 | 60.9 |
| K.F. | F | | 9.54 | 6.20 | 64.9 |
| B.J. | F | | 9.36 | 6.51 | 69.6 |
| R.B. | F | | 10.27 | 6.24 | 60.7 |
| J.B. | F | | 9.27 | 6.18 | 65.8 |
| D.T. | F | | 9.45 | 6.42 | 67.9 |
| | | | | | 60.7 - 71.4** |
| B.S. | M | 23° | 9.89 | 7.48 | 75.6 |
| I.F. | M | | 10.15 | 7.76 | 76.4 |
| T.T. | M | | 9.74 | 7.26 | 74.6 |
| R.T. | M | | 9.84 | 7.33 | 74.6 |
| R.B. | F | | 10.27 | 6.96 | 67.8 |
| B.J. | F | | 9.36 | 6.96 | 74.4 |
| K.F. | F | | 9.83 | 7.26 | 73.8 |
| | | | | | 67.8 - 76.4** |
| W.S. | M | 10° | 10.18 | 6.98 | 68.7 |
| P.C. | M | | 9.94 | 7.44 | 74.8 |
| J.C. | M | | 10.00 | 7.54 | 75.4 |
| P.S. | M | | 9.70 | 6.91 | 71.3 |
| H.F. | M | | 10.30 | 7.92 | 76.8 |
| G.M. | M | | 10.22 | 7.60 | 74.4 |
| A.C. | M | | 9.64 | 7.63 | 79.1 |
| E.L. | M | | 9.53 | 7.57 | 79.4 |
| P.D. | F | | 10.10 | 7.51 | 74.4 |
| J.C. | F | | 10.06 | 7.52 | 74.6 |
| R.D. | F | | 10.30 | 7.78 | 75.5 |
| J.S. | F | | 9.96 | 7.45 | 74.8 |
| D.B. | F | | 10.00 | 7.58 | 75.8 |
| M.N. | F | | 9.40 | 7.22 | 75.8 |
| B.A. | F | | 9.84 | 8.00 | 81.4 |
| R.B. | F | | 10.27 | 7.24 | 70.4 |
| | | | | | 68.7 - 81.4** |

* Values in mg%.
** Range for group.

berg (18) altered pH by the addition of acid or alkali. It has been reported (19) that serum exposed to an excess of $CO_2$ showed a significant increase in ultrafiltrable calcium even though the pH was kept in the physiological range. This increase was thought to represent an artifact owing to the high concentration of carbonic acid. As shown in Table IV, however, pH seems to be the important factor in altering ultrafiltrability of calcium rather than the method of attaining it. The carbon dioxide content of the serum would be quite different if acid were used to lower the pH instead of carbon dioxide, but the ultrafiltrability of the calcium is approximately the same. This substantiates earlier findings (31) that the carbonate binding of calcium is not very strong and contributes only a small amount to the non-protein bound calcium in serum.

*Effect of temperature*

The information concerning any effect of temperature on the ultrafiltrability of calcium is sparse. Among more recent workers, Hopkins, Howard, and Eisenberg (18) have reported that there is no change in the range from room temperature to 36° C. Consequently, all their reported data were obtained at room temperature as were the data of virtually all the earlier workers (Table VIII). Because of the convenience of using a refrigerated centrifuge to maintain a constant temperature, much of the early data in our work on normal serum were obtained at a temperature of 10° C. However, a number of normal values for ultrafiltrable calcium obtained at physiological temperature were noted to be considerably lower than those at 10° C. This suggested the need for investigating the effect of temperature. To reduce the variables to a minimum, ultrafiltrations were carried out simultaneously at several temperatures using serum obtained from one individual. The results are shown in Table V. A large number of other samples were ultrafiltered at 10° C and at 36° C and all the results indicated that the percentage of ultrafiltrable calcium was considerably higher at the lower temperature. The temperature coefficient is approximately 0.5 per cent per degree centigrade.

*Normal values for human serum*

Our individual values for normal healthy young men and women are recorded in Table VI. The

**TABLE VII**

***Repeated determinations of ultrafilterable calcium in normal individuals. Ultrafiltration temperature 36° C***

| Subject | Date | Total Calcium* | Ultrafilterable Calcium* | % Ultrafilterable |
|---|---|---|---|---|
| T.T. (male) | 1/12/56 | 9.26 | 6.38 | 68.9 |
| | 1/19/56 | 9.20 | 6.27 | 68.1 |
| | 2/7/56 | 9.74 | 6.58 | 67.6 |
| | 3/22/56 | 9.71 | 6.64 | 68.5 |
| | 5/10/56 | 10.07 | 7.13 | 70.8 |
| R.T. (male) | 1/17/56 | 9.60 | 6.20 | 64.6 |
| | 1/27/56 | 9.51 | 6.41 | 67.4 |
| | 2/7/56 | 9.84 | 6.44 | 65.5 |
| | 3/22/56 | 9.96 | 6.51 | 65.3 |
| B.J. (female) | 2/9/56 | 9.36 | 6.51 | 69.6 |
| | 3/20/56 | 10.02 | 6.35 | 63.3 |
| | 4/5/56 | 9.52 | 6.30 | 66.2 |
| | 4/9/56 | 9.79 | 6.94 | 70.3 |
| | 5/4/56 | 10.00 | 6.90 | 69.0 |
| | 5/8/56 | 9.73 | 6.85 | 70.3 |
| | 5/31/56 | 9.40 | 6.18 | 66.7 |
| | 7/6/56 | 9.86 | 6.44 | 65.3 |

* Values in mg%.

TABLE VIII

*The per cent of ultrafilterable or diffusible calcium in normal human serum as determined by previous authors*

| Author | Year | Method | pH Control | Temp. | No. of Detns. | Range % |
|---|---|---|---|---|---|---|
| von Meysenbug et al. (2) | 1921 | Compensation Dialysis | Yes | Room | 7 | 67-73 |
| Neuhausen and Pincus (3) | 1923 | Ultrafiltration with positive Hg pressure | No | Room | ? | 40-50 |
| Rona and Melli (4) | 1925 | Compensation Dialysis | Yes | Room | 4 | 64-69 |
| Kirk and King (5) | 1926 | Ultrafiltration with positive $H_2O$ pressure | No | 37°C | 5 | 56-81* |
| Updegraff et al. (6) | 1926 | Morita (37)(combined ultrafiltration and compensation dialysis) | No | Room | 19 | 41-54 |
| Idu (7) | 1927 | Morita-Updegraff | No | Room | 10 | 42-58 |
| Hertz (8) | 1927 | Ultrafiltration with positive $N_2$ pressure | No | Room | 4 | 43-61 |
| Snell (9) | 1930 | Morita-Updegraff | No | Room | 7 | 45-60 |
| Greenberg and Gunther (10) | 1930 | Ultrafiltration with negative pressure | No | Room | 24 | 42-68** |
| McCance and Watchorn (11) | 1931 | Greenberg-Gunther | No | Room | 15 | 44-66 |
| Watchorn and McCance (12) | 1932 | Greenberg-Gunther | No | Room | 6 | 45-59 |
| Nicholas (13) | 1932 | Ultrafiltration with positive $N_2$ pressure | No | Room | 13 | 60-67 |
| Herbert (14) | 1933 | Greenberg-Gunther | No | Room | 7 | 48-58 |
| Benjamin and Hess (15) | 1933 | Ultrafiltration with positive air pressure | No | Room | 21*** | 52 |
| Morison et al. (16) | 1938 | Morita-Updegraff | No | Room | 8 | 43-62 |
| Anning et al. (17) | 1948 | Morita-Updegraff | No | Room | 20 | 51 |
| Hopkins et al. (18) | 1952 | Lavietes(28) (ultrafiltration over Hg) | Yes | Room | ? | 65-75 |
| Prasad and Flink (19) | 1955 | Ultrafiltration with centrifugal force | Yes | Room | ? | 47-56# 57-72 |

* Questionable total serum calciums.
** Assuming total serum calciums of 10 mg%.
*** Include 17 infants.
# Value depending upon method of collection and equilibration of serum.

mean values for the per cent of ultrafiltrable calcium determined at 36° C is 65.9 per cent while at 10° C it is 75.2 per cent. At 23° C the mean is 73.9 per cent but we believe this is erroneously high due to the small number of samples determined at this temperature. No sex difference was observed. In Table VII are recorded determinations of ultrafiltrable calcium on three normal individuals, extending over a period of several months. It can be seen that fluctuations occurred over the period and these were all within the normal range (Table VI). Repeated determinations

of ultrafiltrable calcium in human subjects during a single day, however, are remarkably constant (32).

In Table VIII is recorded the per cent ultrafiltrability or diffusibility of the calcium of normal human serum calculated from the data of previous authors. It is immediately apparent that, with the exception of von Meysenbug, Pappenheimer, Zuker, and Murray (2), Rona and Melli (4), Nicholas (13) and Kirk and King (5), earlier workers obtained results significantly lower than our values and those of the two most recent authors (18, 19).

From the previous discussion on the effect of pH on the quantity of ultrafiltrable calcium it seems most probable that failure to control the pH during ultrafiltration was predominantly responsible for the consistently lower values obtained by these early workers. In this connection, it is of interest that the results of von Meysenbug, Pappenheimer, Zuker, and Murray (2) and of Rona and Melli (4) were obtained by compensation dialysis in a closed system where the pH was probably maintained constant throughout the procedure. The original Rona and Takahashi investigations of 1911 (1) were done by essentially the same method. Their value for the diffusible serum calcium of various domestic animals was between 65 per cent and 75 per cent! Nicholas (13) and Kirk and King (5) took no special precautions regarding pH control but the apparatus they used was closed during the entire filtration and positive pressure was maintained over the serum with nitrogen or air.

## DISCUSSION

Our data indicate that, with a normal total serum calcium concentration of about 10 mg. per cent, 6 to 7 mg. per cent of this calcium is ultrafiltrable. McLean and Hastings (22), by their frog heart technique, concluded that about 1.3 mM per L. (5.2 mg. per cent) of "ionic" calcium exists in normal serum. Calculations based on the actual normal serum concentrations of citrate, phosphate, and bicarbonate, activity coefficients, and the reported pK's of the calcium complexes of these anions reveal that about 0.3 mM per L. (1.2 mg. per cent) of calcium exists in normal serum in the form of these known complexes (33). The sum of these two values, 6.4 mg. per cent, is remarkably similar to the mean value of our experimentally determined range for normal total ultrafiltrable calcium and closely approaches the value for "biologically active" calcium suggested by the data of Yendt, Connor, and Howard (21).

Variations in the pH of the blood in human beings *in vivo* seldom extend more than 0.2 pH units above or below the 7.35 to 7.40 range even in pathological states involving alterations in acid-base balance. From our data this would mean only a minor alteration in the actual quantity of ultrafiltrable calcium, well within the 10 per cent range found in normal human subjects. In acute conditions, however, such as hyperventilation tetany, a sudden minor shift in the quantity of available calcium may well be highly important. It has been reported that in hyperventilation tetany, total serum calcium and total ultrafiltrable calcium do not change significantly (34, 35). However, the actual alterations in the quantity of calcium involved due to pH changes in the physiological range are small enough so as to lie near or within the limits of accuracy of all the experimental methods for estimating total and ultrafiltrable calcium.

The observed effect of temperature on the quantity of ultrafiltrable calcium in human serum was somewhat surprising. Most complexes become more stable at lower temperature, but we found that more calcium was ultrafiltrable as the temperature at which the procedure was carried out was decreased.

The only bits of corroborative evidence for this temperature effect we were able to find were in the work of McLean and Hastings (22), Lavietes (28), and Marrack and Thacker (26). In 1926, the latter authors, by dialysis of protein solutions at room temperature and at 37° C, concluded that values for non-diffusible calcium of serum would be lower if the determinations were made at room temperature. Lavietes, in 1937, described his anaerobic ultrafiltration apparatus and referred to the fact that the quantity of calcium filtered varied with temperature but no data were given. McLean and Hastings noted that the amount of "ionic calcium" was about 0.15 mM per L. (0.6 mg. per cent) higher at 15° C than at 25° C. Although questioning the significance of this observation, they did calculate by extrapolation that calcium ion concentrations would be 0.8 mg. per cent lower

at 38° C than those observed at room temperature if this temperature effect were real. That is, with an average normal serum calcium of 10 mg. per cent the concentration would be about 8 per cent lower. This value agrees very well with our data on normal values at different temperatures recorded in Table V.

The exact mechanism responsible for this temperature effect is not clear. It may be that in the binding of calcium to protein a weaker bond is obtained at lower temperatures, but Katz and Klotz (36) claim that the binding of calcium to bovine serum albumin is independent of temperature from 0° C to 25° C. A change in pH is apparently not involved. We have found that the pH in an aliquot of serum measured at room temperature, after 5 per cent $CO_2$–95 per cent $O_2$ equilibration at that temperature, did not change significantly when the pH was remeasured after heating to 36° C.

One other possible explanation for the phenomenon of decreased protein-bound calcium at the lower temperatures is suggested when one considers the binding of calcium by other anions in serum such as citrate, phosphate, and bicarbonate. The relationship between the latter and ionic calcium and calcium bound to protein can be expressed by the equation $CaX \rightleftharpoons Ca^{++} \rightleftharpoons CaProt$ where X refers to these ultrafiltrable anionic complexers of calcium. If the temperature coefficients of the filtrable complexes of calcium were greater than those of the protein complexes, the net effect of a lowering of temperature would be to have more of the calcium in the form of these ionic complexes and a resultant increase in ultrafiltrable calcium as found. The data of Katz and Klotz (36), who found that the binding of calcium ions by bovine serum albumin was independent of temperature, lend support to this explanation. If this explanation is correct, a net *decrease* in the actual ionic form of calcium would occur as the ultrafiltration temperature is lowered although total ultrafiltrable calcium increases. That is, more of the calcium ultrafiltered would be in an associated form. Thus, it would appear that the McLean and Hastings frog heart method, which also responds to a lowering of temperature with an apparent increase in "ionic" calcium, measures more than just the ionic calcium in the test solution. It is clear that further work with pure proteins is necessary to elucidate the exact nature of the temperature effect observed.

## SUMMARY

1. A new simple ultrafiltration apparatus is described and the procedure for the ultrafiltration of human serum is outlined.

2. The importance of the method of sample collection, duration of filtration, pH and temperature was investigated. The ultrafiltration results were affected considerably by pH and temperature.

3. For healthy human subjects, the range for ultrafiltrable calcium at physiological pH and temperature was found to be 60 to 70 per cent of the total serum calcium. The results of previous authors are reviewed and compared with the present findings.

4. The quantitative distribution of the various calcium fractions in human serum and the significance of the observed effect of pH and temperature on the ultrafiltrability of calcium are discussed.

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# A AND B ANTIGENS IN THE HUMAN EPIDERMIS[1]

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Several attempts to demonstrate the presence of A and B antigens in the human epidermis have been undertaken in the past, but with negative results (1, 2). Recently, Coombs, Bedford, and Rouillard (3) proved by the mixed agglutination method the existence of A and B group specific substances in epidermal cells.

In continuation of studies on the A and B antigens in the human cornea (4, 5) the presence of these antigens in the human epidermis was investigated.

The following is a report of this investigation.

## MATERIAL AND METHODS

Thin split thickness skin was obtained by means of an electric dermatome from 19 patients, selected at random, who were admitted to the hospital for various skin grafting procedures. No differentiation was made between secretors and non-secretors. Part of this skin was used for skin grafting, the rest rinsed in physiological saline and despatched to the laboratory, completely immersed in a container full of saline. There the excised skin was cut to standard size, $3 \times 3$ cm. and washed 3 times in 200 ml. normal saline. The flask containing saline and skin was agitated vigorously on a Kahn shaking machine for 10 minutes. In order to test for complete removal of erythrocytes, the last saline washing was centrifuged in large conical tubes and the minute sediments examined microscopically for red blood cells. If only one erythrocyte was found, three additional washings were carried out. Every sediment was further tested by the Benzidine Reaction (6). This was sometimes found to be positive after the first washing, but always negative after the third. After this treatment the skin was used either fresh or frozen at $-20°$ C in 2 ml. of normal saline. For every experiment $0.5 \times 0.5$ cm. pieces of skin were cut into very thin slices with the aid of fine scissors and scalpels. These slices were homogenized for 30 minutes in a small pyrex glass tissue grinder with a pyrex glass pestle connected to a variable speed stirring apparatus. Five-tenths ml. of saline was added to the homogenate during homogenization. To prevent excessive heat production the grinder was immersed in an ice bath and the stirring carried out at intervals. The fine white pasty mass obtained served as antigenic material.

*Preparation of sera.* Sera were inactivated at 56° C for 30 minutes, and titrated for anti-A and anti-B isoagglutinins. Only sera with the minimal titer of 1:128 when undiluted were used for absorption experiments. Sera were distributed in small quantities stored at $-20°$ C until used; no serum was frozen and thawed more than once.

*Isoagglutinin determination.* Titrations of isoagglutinins were performed by the double serial dilution method against freshly prepared 2 per cent erythrocyte suspensions. Results were read after allowing the tubes to stand at room temperature for 30 minutes. The titer was expressed as the last dilution at which agglutinates could be detected by the naked eye. The tubes were neither centrifuged nor examined microscopically. All titrations were performed by the same technician, and the results read separately by two different persons (D. N. and J. G.). Changes in the intensity of agglutination were not taken as an indication for a reduction in titer, only the complete disappearance of agglutination was assumed to prove real absorption.

## EXPERIMENTAL PROCEDURES AND RESULTS

### *Absorption experiments*

Absorption experiments were performed in siliconized tubes to prevent the adherence of epidermal material to the glass. Five-tenths ml. of the homogenate was added to 0.5 ml. of high-titred A, B or O serum. Undiluted sera or sera diluted with normal saline in a proportion of 1:1 were used in the absorption experiments. The skin serum mixture was left for 8 hours at a temperature of 6° C and was shaken every 30 minutes. After 8 hours the serum was separated by centrifugation at 3,000 r.p.m. for 10 minutes.

Nineteen absorption experiments were performed with split skin homogenates from individuals of blood groups A, B, O and AB, 4, 3, 6 and 6 instances of each, respectively. When O serum was absorbed by epidermal homogenates from a donor of blood group A or from a donor

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TABLE I

*Results of absorption and elution experiments*

| Experiment No. | Titer of serum O before absorption | | Epidermal material from donor of blood group | Titer of serum O after one absorption with epidermal homogenate | Titer of eluate against red blood cells |
|---|---|---|---|---|---|
| 1 | Anti-A | 1:128 | A | 1:64 | 1:8 |
| | Anti-B | 1:256 | | 1:256 | 0 |
| 2 | Anti-A | 1:128 | A | 1:64 | 1:8 |
| | Anti-B | 1:256 | | 1:256 | 0 |
| 3 | Anti-A | 1:64 | A | 1:16 | 1:4 |
| | Anti-B | 1:128 | | 1:128 | 0 |
| 4 | Anti-A | 1:64 | A | 1:8 | 1:8 |
| | Anti-B | 1:128 | | 1:128 | 0 |
| 5 | Anti-A | 1:128 | B | 1:128 | 0 |
| | Anti-B | 1:256 | | 1:128 | 1:16 |
| 6 | Anti-A | 1:64 | B | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:32 | 1:8 |
| 7 | Anti-A | 1:64 | B | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:32 | 1:4 |
| 8 | Anti-A | 1:128 | AB | 1:64 | 1:8 |
| | Anti-B | 1:256 | | 1:128 | 1:4 |
| 9 | Anti-A | 1:128 | AB | 1:32 | 1:4 |
| | Anti-B | 1:256 | | 1:128 | 1:4 |
| 10 | Anti-A | 1:64 | AB | 1:16 | 1:2 |
| | Anti-B | 1:128 | | 1:64 | 1:2 |
| 11 | Anti-A | 1:64 | AB | 1:16 | 1:2 |
| | Anti-B | 1:128 | | 1:64 | 1:2 |
| 12 | Anti-A | 1:64 | AB | 1:16 | 1:4 |
| | Anti-B | 1:128 | | 1:32 | 1:2 |
| 13 | Anti-A | 1:64 | AB | 1:32 | 1:4 |
| | Anti-B | 1:128 | | 1:16 | 1:4 |
| 14 | Anti-A | 1:128 | 0 | 1:128 | 0 |
| | Anti-B | 1:256 | | 1:256 | 1:1 |
| 15 | Anti-A | 1:64 | 0 | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:128 | 0 |
| 16 | Anti-A | 1:64 | 0 | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:128 | 0 |
| 17 | Anti-A | 1:64 | 0 | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:128 | 0 |
| 18 | Anti-A | 1:64 | 0 | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:128 | 0 |
| 19 | Anti-A | 1:64 | 0 | 1:64 | 0 |
| | Anti-B | 1:128 | | 1:128 | 0 |

of blood group B, only the titer of the corresponding isoagglutinin was reduced, while that of the other agglutinin remained unchanged. When O sera were absorbed by epidermal homogenates of AB donors, absorption of both anti-A and anti-B isoagglutinins occurred. Similar results were obtained when A and B sera were absorbed by epidermal homogenates of the corresponding group. When epidermal material of group O donors was used for the absorption experiments, no reduction whatsoever could be detected in either the anti-A or anti-B titer of the absorbed serum (see Table I).

In three cases, repeated absorptions were performed with epidermal material from individuals of the same blood group. After three consecutive absorptions the titer of the antiserum was reduced from 1:128 to 1:4.

Table II summarizes the results of one such repeated absorption experiment.

*Elution experiments*

Eluates were prepared from the homogenates which served for the absorption experiments. The absorbates were washed three times in large amounts of saline to ensure removal of traces of serum. The washed epidermal sediment was resuspended in 0.4 ml. of normal saline and vigorously shaken by hand in a water bath of 56° C for 5 minutes. It was then centrifuged at 3,000 r.p.m. for 1 minute in cups jacketted with water at 56° C and the eluate was immediately removed. Using the above technique, eluates of 19 different samples of split skin homogenates were prepared and examined for agglutinating capacity against erythrocytes. In all cases eluates agglutinated erythrocytes of the corresponding group.

Results of absorption and elution experiments are summarized in Table I. Apart from the quantitative reduction of the titer a marked difference in the intensity of agglutination in the last two to three tubes of the titrations was observed.

## DISCUSSION

Recent studies on skin homografts have established the fact that human epidermis is antigenic and may induce the production of antibodies (1, 7–10). The question of which antigens are concerned in the homograft reaction is still unsettled.

The possible existence of A and B antigens in human epidermis was studied for the first time in 1928 by Yosida (2), with negative results. Medawar (11) in 1946 examined the possibility that a

TABLE II

*Results of three consecutive absorptions of A serum with epidermis of an individual of blood group B*

| Titer of serum | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 |
|---|---|---|---|---|---|---|---|---|
| Before absorption | +++ | +++ | +++ | +++ | +++ | ++ | + | ± |
| After first absorption | +++ | +++ | +++ | ++ | ++ | ± | − | − |
| After second absorption | +++ | +++ | ++ | + | − | − | − | − |
| After third absorption | ++ | + | ± | − | − | − | − | − |

prior blood transfusion leads to immunity to skin grafts from the same animal. He also investigated the question of whether the transplantation of homologous skin results in the formation of red cell agglutinins. He could not demonstrate an antigenic relationship between erythrocytes and epidermis. In 1954, Co Tui, Campbell, Rothburd, and Fand (1) studied the same problem with the aid of tissue cultures. They used skin cultures from human donors of A and B blood groups and treated them with antisera; they were, however, unable to observe morphologic changes in the explants.

Recently, Coombs, Bedford, and Rouillard (3) demonstrated, by mixed agglutination between erythrocytes and epidermal cells, that human epidermal cells possess A and B antigens. Gorer (12) mentions the finding of immune anti-A antibodies in two patients after skin grafting. Clarkson and Gorer (13) reported a case of a burnt child who, following a skin homograft, developed immune anti-erythrocyte antibodies. Pfeffer and Rogers (14) stated that they observed less violent rejections of the homotransplanted skin grafts when using homografts from donors of compatible blood groups.

In the present investigation the existence of A and B antigens has been established by absorption and elution methods. Split skin homogenates from individuals of blood groups A and B reduced the titer of anti-A and anti-B sera. The eluates prepared from these homogenates agglutinated red blood cells of the corresponding group. It may be assumed that the presence of A and B antigens in the human epidermis will be of importance in skin homografting.

## SUMMARY

Human epidermis was found to contain A and B blood group antigens corresponding to those of red blood cells.

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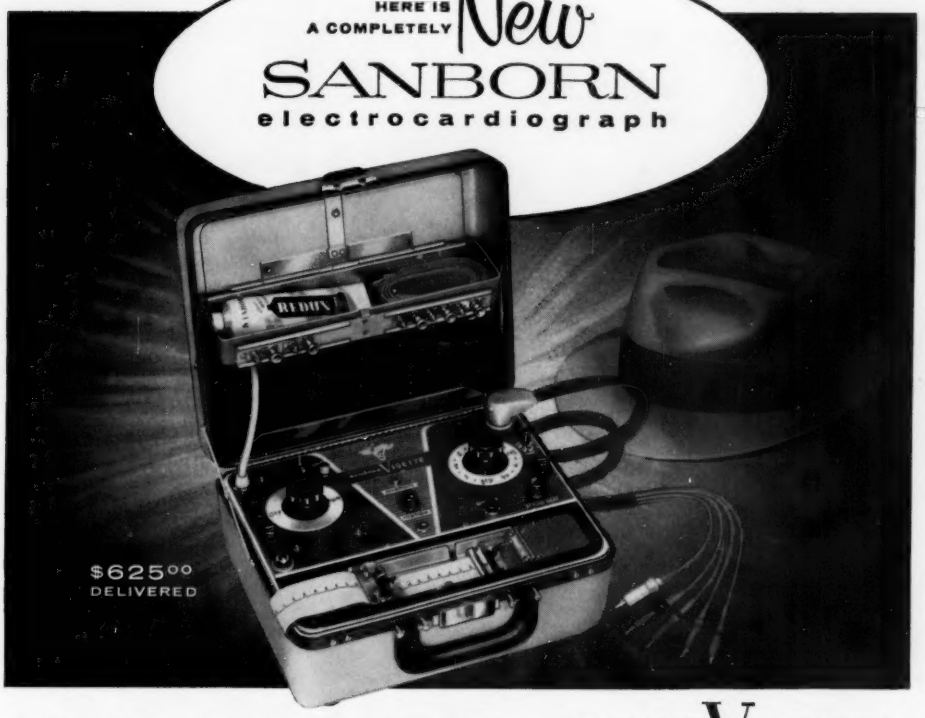


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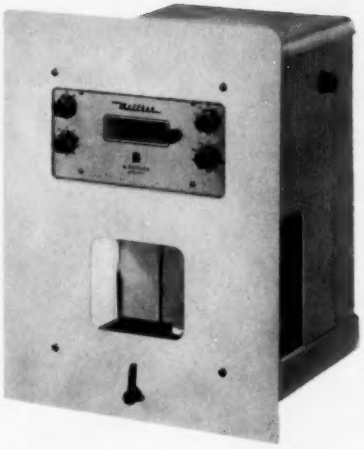

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